

Gwynne Attarian, et al.
Application No.: 09/747,385
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Gwynne Attarian, et al.

Application No.: 09/747,385

Filed: December 22, 2000

For: FUSOBACTERIUM NUCLEIC
ACIDS, PLASMIDS AND VECTORS

Customer No.: 20350

Confirmation No. 9499

Examiner: Lambertson, David A.

Technology Center/Art Unit: 1636

Declaration of Susan A. Kinder Haake
Under 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Susan A. Kinder Haake, being duly warned that willful false statements and the like are punishable under 18 U.S.C. 1001, by fine or imprisonment or both, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true.
2. I am a co-inventor of the invention disclosed and claimed in the above identified patent application. I have read and am familiar with the contents of the subject patent application.
3. I hold a Ph.D. (1993) in Microbiology from University of Texas, San Antonio, a D.M.D. (1979) from Tufts University, a Certificate in Periodontology (1985) and a M.Dent.Sc. (1985) from the University of Connecticut, and a Bachelor of Arts (1976) from Hamilton-Kirkland College. I am an Associate Professor of Dentistry, Sections of



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P.O. Box 1450
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Confirmation No.

Examiner: Davis, Katharine F.

Technology Center/Art Unit: 1636

STATEMENT UNDER 37 C.F.R. §1.804

Post-it® Fax Note	7671	Date 4/6	# of pages ▶ 2
To Elisabeth Sampson		From Susan K. Haake	
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Sir:

I, Susan A. Kinder-Haake, am a named inventor on the above-referenced patent application and have control over the conditions of the deposit described herein.

I hereby confirm that the following biological deposits:

ATCC PTA-5816 (cell line comprising pFN2), and

ATCC PTA-5815 (cell line comprising pFN3)

referred to in the above referenced application, were deposited on February 13, 2004 with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110, where the deposits were granted the accession numbers PTA-5815 and PTA 5816. The deposit was made pursuant to the provisions of the Budapest Treaty and the viability of the deposit was tested on February 20, 2004. A copy of the Deposit Receipt and Viability Statement is attached hereto as Appendix A.

I also confirm that, subject to paragraph (b) of 37 C.F.R. §1.804, the deposited material is the same as that specifically identified in the above referenced patent application and was in Applicants' possession at the time the application was filed.

Respectfully submitted,



Susan A. Kinder Haake D.M.D. Ph.D.

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Attachments

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Periodontology and Oral Biology and Medicine, and a member of the Dental Research Institute at the University of California Los Angeles.

4. My field of expertise is in molecular microbiology, including the characterization and use of bacterial plasmids in developing genetic transfer systems.

5. I believe that one skilled in the art of molecular biology would regard proteins with 90% sequence identity to SEQ ID NO:1 (repA) as functionally equivalent to the repA protein of the invention. Furthermore, I believe an individual skilled in the art would be able to identify such repA proteins given the teachings of the present specification in combination with the literature available at the time the present application was filed. Indeed, based on current knowledge of plasmid replicons, 90% is probably a very conservative estimate of the amount of sequence identity needed to assure functionality (*i.e.*, function would probably be conserved with even greater alteration of the amino acids).

6. **Related Replication initiator proteins from theta-replicating plasmids of the iteron-containing class may exhibit more than 75% amino acid sequence divergence.**

The RepA protein (SEQ ID NO:1) and iteron sequences (as exemplified by SEQ ID NO:3) of the invention comprise *Fusobacterium* plasmids that are members of a group of plasmids known as "theta-replicating plasmids of the iteron-containing class". The conclusion that pFN1 is a member of this class of plasmids is based on DNA sequence analysis of the replicon structure (*i.e.*, presence of iterons) and homology of the Rep protein with those of other theta-replicating plasmids. The features common to this class of plasmids are discussed in the accompanying del Solar review paper (Exhibit A).

The del Solar paper (Exhibit A) presents a comparative amino acid sequence analysis of the replication initiator proteins from theta-replicating plasmids of the iteron-containing class. This analysis, while not addressed specifically to the pFN1 replicon, is nonetheless applicable to the pFN1 replicon as the Rep proteins analyzed in Exhibit A are all functional components of theta-replicating plasmids with origin structures comparable to that of the fusobacterial plasmid pFN1.

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The analysis presented in Exhibit A shows that replication initiator proteins such as RepA, typically exhibit much greater than 10% sequence divergence *i.e.* considerably less than 90% sequence identity. For example, comparison of the two most divergent members of the family of related replication initiator proteins, pPS10 and R6K, shown in Figure 2, on page 439 of Exhibit A, reveals that related replication initiator proteins from theta-replicating plasmids of the iteron-containing class may exhibit more than 76% amino acid sequence divergence *i.e.* less than 24% sequence identity. This percentage is quite striking, particularly if one considers that this calculated sequence identity between pPS10 and RK6 Rep proteins is based only on amino acids that are in *aligned* portions of the two proteins (in Figure 2, there are 223 aligned residues, of which 53 are identical). Unaligned portions of the two proteins *i.e.*, the additional segments of amino acids of the R6K protein lacking a corresponding amino acid in the pPS10 protein, are *not* taken into account in this calculation. Thus, in essence this means that the 24% identity figure *overstates* the identity.

To underscore the sequence variation possible among different, related replication initiation proteins, I calculated the percent sequence identity between the full length replication initiator proteins of plasmids pPS10 and RK6 using the sequences cited in the legend to Figure 2 panel A, in Exhibit A. I did a ClustalW alignment of the entire protein predicted amino acid sequence. For the full length amino acid sequences, a comparison of pPS10 versus R6K reveals that these related replication initiator proteins are 17.14% identical, 20% different but strongly similar, 7.62% weakly similar, and 55.24% different. Thus, in simple terms of identity versus non-identity, the two related replication initiator proteins are 17.14% identical and 82.86% non-identical.

7. In a family of related lactococcal plasmids, replication initiator proteins with 60 to 80% amino acid sequence identity are able to complement one another's function.

Another demonstration that related replication initiator proteins from theta-replicating plasmids of the iteron-containing class exhibit wide sequence divergence while retaining similar functionality is provided by the accompanying Seegers *et al.* research paper

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(Exhibit B). Seegers *et al.* show that in a family of related lactococcal plasmids, replication initiator proteins with only 60 to 80% amino acid sequence identity are able to complement one another's function. Thus, the proteins are functionally equivalent. Again, the relevance to the case under consideration is that these lactococcal plasmids are all theta-replicating plasmids with origin structures comparable to that of the fusobacterial plasmid pFN1.

8. Based on the data presented above, I believe that in fact much more than 10% of the amino acids in the repA sequence could be altered and the function of the repA replication protein would still be preserved. Furthermore, I believe that one of skill in the art would be able identify such functional repA proteins, without undue experimentation, based on the teachings of the specification in combination with the knowledge in the art at the time the application was filed.

The Declarant has nothing further to say.

Dated: 3/4/04

By: Susan A. Kinder Haake

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Replication and Control of Circular Bacterial Plasmids

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INTRODUCTION

Plasmids are extrachromosomal DNA elements with characteristic copy numbers within the host. These replicons have been found in species from the three representatives of the living world, namely, the domains *Archaea*, *Bacteria*, and *Eukarya* (318). Plasmids may constitute a substantial amount of the total genetic content of an organism, representing more

than 25% of the genetic material of the cell in some members of the *Archaea* (127, 331). They can incorporate and deliver genes by recombination or transposition, thus favoring genetic exchanges in bacterial populations. Since plasmids can be introduced into new hosts by a variety of mechanisms, they can be considered to be a pool of extrachromosomal DNA which is shared among populations. The wealth of genetic information carried by plasmids, their impact in the microbial communities, and the potential of these elements to act as natural cloning vectors have stimulated research into plasmids not only from the fundamental but also from the clinical, biotechnological, and environmental points of view. Three main factors have

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contributed to the development of plasmid research: (i) the genetic organization of these elements is apparently simple, (ii) they can be easily isolated and manipulated in vitro, and (iii) since plasmids are dispensable, their manipulation does not appear, in principle, to have adverse consequences to the hosts.

The feature that better defines plasmids is that they replicate in an autonomous and self-controlled way. The analysis of plasmid replication and its control has led to milestone discoveries, such as the existence of antisense RNAs, and has contributed to the unraveling of mechanisms of DNA replication, macromolecular interactions, and control of gene expression. The ability of some plasmids to pass across the so-called genetic barriers among different living organisms has posed questions about general mechanisms governing replication and about the communication between plasmid replication components and the host machinery involved in DNA replication. This plasmid-host communication has attracted the attention of researchers working in environmental and in evolutionary fields. Plasmid host range studies also have clear implications in clinical microbiology and in biotechnology. Despite their autonomous replication, plasmids extensively use the replication machinery of the host, and therefore plasmid replication studies facilitate the exploration of the mechanisms involved in chromosome replication.

PLASMID REPLICATION MECHANISMS

There are three general replication mechanisms for circular plasmids, namely, theta type, strand displacement, and rolling circle (RC). Historical development of research on plasmids has led to the idea that theta replication is more frequent in replicons from gram-negative than from gram-positive bacteria whereas the opposite is found for plasmids replicating by the RC mode. This belief is probably wrong. It is true, however, that present knowledge on theta-replicating plasmids stems from replicons from gram-negative bacteria and that on RC-replicating plasmids derives from replicons from gram-positive hosts. Strand displacement replication has been associated with broad-host-range plasmids from the IncQ family. The molecular interactions and the functional relationships that take place in these three types of replication mechanisms are the focus of this review. Linear plasmids have been found in both gram-positive and gram-negative bacteria, and their structure can be of two types: those having a hairpin at each end, and those having a protein covalently bound at their 5' ends. Linear plasmids of the first group replicate via concatemeric intermediates, whereas those of the second group seem to replicate by a protein-priming mechanism, similar to that of bacteriophage ϕ 29 (264). However, initiation of replication from an internal origin in a plasmid with a terminal protein has been reported (48). Linear plasmids have been reviewed previously (123), and they will not be discussed here. Replication of plasmids from gram-negative bacteria has been specifically addressed (168a).

Concerning their genetic structure, plasmids have an essential region which contains the genes or loci involved in replication and its control. The organization of this essential region corresponds, in general, to the one described in the replicon model. In addition, plasmids may bear genes that could be considered dispensable, although they could actually play an important role for the plasmid itself and/or for the host. Some of these so-called dispensable genes are involved in processes such as plasmid transfer and spread among bacteria, resistance to antibiotics and heavy metals, resistance to radiation, and transfer of DNA to higher eukaryotes. Within the plasmid

essential region, several genes and sequences can be considered. (i) The first is the origin(s) of replication (generically termed *ori*), which is characteristic of each replicon. (ii) Although this is not a general feature, many plasmids encode a protein involved in the initiation of replication, usually termed Rep protein. (iii) The third is the plasmid-borne genes involved in the control of replication. The requirement of a plasmid-encoded initiator is reflected by the presence of DNA cognate sites in the origin of replication, where protein-DNA interactions take place. These specific sites are the hallmark of a class of replicons that are different from replicons that do not require specific initiators.

Replication by the Theta Mechanism

Replication by the theta-type mechanism has been most extensively studied among the prototype circular plasmids of gram-negative bacteria, although this replication mode has also been described for plasmids isolated from gram-positive bacteria, namely, the streptococcal/enterococcal Inc18 group (40), some lactococcal replicons (152), and at least one *Bacillus subtilis* plasmid (192). DNA replication through the theta mechanism involves melting of the parental strands, synthesis of a primer RNA (pRNA), and initiation of DNA synthesis by covalent extension of the pRNA (163). DNA synthesis is continuous on one of the strands (leading strand) and discontinuous on the other (lagging strand), although synthesis of the two strands seems to be coupled (reviewed in references 148 and 326). Theta-type DNA synthesis can start from one or from several origins, and replication can be either uni- or bidirectional. Under electron microscopy (EM), the replication intermediates are seen as typical Θ ("theta")-shaped molecules that, when digested with enzymes that cleave within the replicated region, yield Y-shaped molecules ("forks"). The replication intermediates can also be monitored by one- or two-dimensional electrophoresis. These analyses provide information on the nature of the replication intermediates, direction of replication, location of the origin and terminus, and degree of coupling between leading- and lagging-strand synthesis.

With some exceptions, plasmids using the theta mechanism of replication require a plasmid-encoded Rep initiator protein. Some replicons may require the host DNA polymerase I (DNA Pol I) during the early stages of leading-strand synthesis. Some features of various well-known replicons which are described here are depicted in Fig. 1.

Origins of replication. Plasmid origins of replication can be defined as (i) the minimal *cis*-acting region that can support autonomous replication of the plasmid; (ii) the region where DNA strands are melted to initiate the replication process, or (iii) the base(s) at which leading-strand synthesis starts. Replication origins contain sites that are required for interactions of plasmid-encoded and/or host-encoded proteins.

(i) General features. With some exceptions, initiation of plasmid DNA replication requires a specific plasmid-encoded Rep initiator protein. This is reflected by the presence, at the origin of replication, of specific sequences with which the Rep protein interacts. Additional features found in many origins of theta-replicating plasmids are (i) an adjacent AT-rich region containing sequence repeats, where opening of the strands and assembly of host initiation factors occur, and (ii) one or more sites (*dnaA* boxes) where the host DnaA initiator protein binds (30, 163). Multiple Dam methylation sequences, which are present in the origin of replication of the *Escherichia coli* chromosome, *oriC*, can also be found at the origin of replication of plasmids such as P1 (36, 38) and pSC101 (30). Methylation is

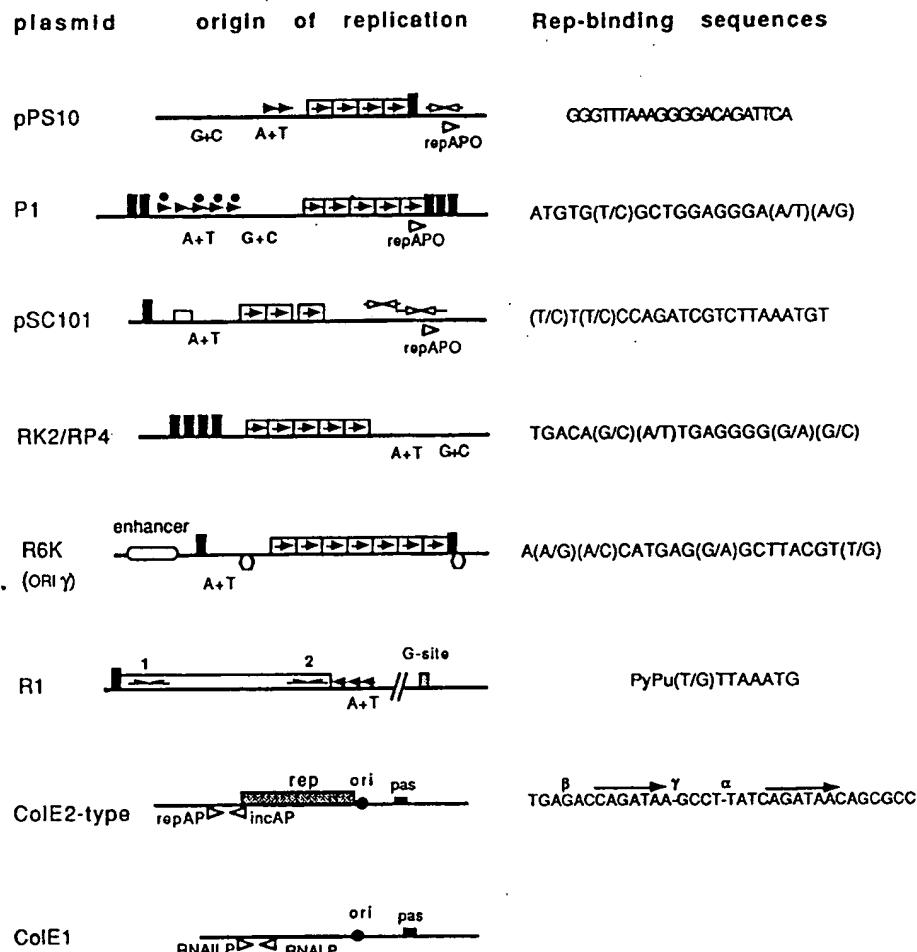


FIG. 1. Origins of replication and related regions of some representative theta-replicating plasmids from gram-negative bacteria. Plasmid names (left), origins of replication (center), and Rep initiator-binding sites (right) are indicated. The symbols used are as follows: solid boxed arrows correspond to repeated Rep-binding sequences (iterons); open arrows above maps indicate inverted repeats that have partial homology to the iterons; solid arrowheads indicate repeats found in AT-rich regions (A+T); for R1, arrows indicate the imperfect palindromes initially recognized by the RepA initiator protein. Promoters are indicated as open arrowheads below the maps. Other sites of interaction are as follows: IHF-binding sites (open rectangles), *dnaA* boxes (solid rectangles); FIS-binding sites (hexagons), *dam* methylation sites (solid circles), and primase assembly sites (pas). Other sites are indicated in the figure. Maps are based on the following references: pPS10 (104, 215); pSC101 (132, 284); P1 (6); RK2 (278); R6K (277); R1 (101); ColE1 (280, 301); ColE2-type plasmids (124).

not essential for replication, its role being primarily in postreplication (3). Dam methylation sequences are not present in other plasmid replicons.

Comparative analysis of the structural organization of the Pol I-independent origins of replication predicts that although the Rep-binding site is located within a potentially curved DNA region, the DNA within the repeats of the AT-rich region is essentially straight (81). Intrinsic DNA bends at the Rep-binding sites would favor additional curvatures of the origin induced by Rep proteins. The origins of replication can also contain sites for factors (e.g., the integration host factor, IHF, or the factor for inversion stimulation, FIS) that play an architectural role. These host-encoded proteins favor a topological proximity between different *ori* regions or even between different origins present in the same plasmid (as in plasmid R6K [see below]). The plasmid DNA sites are essential components of the origin of replication since they are required to organize a functional replisome (61, 62, 282). The presence of DNA sites for the binding of structural factors, found at the origin of replication of several plasmids (see below), resembles the situation found in *oriC* (317).

(ii) **Itron-containing origins.** In many cases, the origin of replication contains directly repeated sequences, termed iterons, which are the binding sites for the plasmid-encoded Rep proteins and which have control properties. As discussed below, iterons not only are essential for replication but also are key elements for the control of plasmid replication (reviewed in references 51, 87, 155, and 223). Among plasmids which restrict their establishment to a single or a few species of enterobacteria, iterons have been described for several replicons like P1 (5), F (209, 295), pSC101 (52), R6K (97, 98, 277, 278), Rts1 (144), and pColV-K30 (247). Iterons are also found in theta-replicating broad-host-range plasmids such as RK2/RP4 (241, 279), pCU1 (164) and pSa (286), as well as in conditional broad-host-range plasmids such as pPS10 (85, 104, 215). It should be noted that the presence of directly repeated sequences to which Rep proteins bind is not restricted to plasmids replicating by the theta mechanism, since these sequences have been reported for plasmids using the strand-displacement mechanism or the RC mechanism (171, 176, 267). Iterons can also be found outside the origin region in some plasmids (P1, F, RK2, R6K, Rts1, and pColV-K30). These iterons, unlike

the origin iterons, are not required for initiation but play an important role in the control of replication, as the origin iterons do. In plasmids that do not have auxiliary iterons, the origin iterons are the only locus involved in control (see below).

Iterons can be adjacent or separated by intervening sequences. Iterons found in the origin region tend to be arranged as tandem repeats situated at a distance that is, in general, a multiple of 11 bp, i.e., close to the helical periodicity of the DNA double helix. This implies that the Rep-iteron nucleoprotein complexes roughly place the Rep molecules aligned on the same face of the DNA. In general, for a particular origin, the sequences of the different iterons are not identical, although they adjust to a consensus motif that defines the essential features of these sequences. However, the four 22-bp iterons of plasmid pPS10 are identical (215). Statistical analysis of the frequency of base changes within the iterons of plasmid P1, combined with the available footprinting data, have been performed (242). Three highly conserved sequence patches are found within the iterons of this replicon. The two outer patches are separated by one helix turn. Protection experiments indicated that the major groove sides of those patches are contacted by the RepA initiator protein of P1. The function of the middle patch is less clear, but it may contribute to a proper conformation of the RepA-binding site. It is remarkable that this pattern resembles the DNA-binding patterns of dimeric proteins, some of which are transcriptional repressors. Taking into account that some of these iterons are contacted by monomeric forms of the initiator proteins, this may reflect the presence of two DNA-binding domains in RepA (discussed in reference 51), a feature that may be extended to other plasmid-encoded Rep proteins (100). Alignment of iterons present in the origin of replication of different plasmids showed the conservation of the hexanucleotide TCAGPuG (86), which is directly involved in the binding of the π initiator protein to the *ori- γ* region of plasmid R6K (97, 98).

Multiple iterons are required for origin activity, although not all iterons present in a given origin have to be essential. For instance, removal of one of the seven iterons from *ori- γ* of plasmid R6K has no effect but deletion of two reduces the efficiency of replication and deletion of three or more abolishes plasmid replication (160). Interestingly, the deletions make *ori- γ* replication independent of DnaA (16a). In the case of P1, all five iterons seem to be required for replication in vivo, but deletion of one can be tolerated in an in vitro replication system (314).

Single iterons are present in the *ori- α* and *ori- β* origins of plasmid R6K and in the minimal origins of plasmids ColE2 and ColE3. In R6K, *ori- α* and *ori- β* contain just one iteron and half an iteron, respectively (87). This situation is compensated for by the presence of a *cis*-acting sequence (enhancer), which is located in a third origin (*ori- γ*) that contains seven iterons. The enhancer facilitates the transfer of the initiator π protein, assembled at the seven iterons of *ori- γ* , to *ori- α* and *ori- β* , and leads to initiation of DNA replication (see below). The smallest of all the prokaryotic origins described so far have been found in the ColE2 and ColE3 replicons (322). They consist of a stretch of 47 bp (ColE2) or 33 bp (ColE3) and contain two major directly repeated sequences.

(iii) Other origin configurations. Origins of replication without iterons can be found in other well studied theta-replicating plasmids like R1 and ColE1, as well as in plasmid pLS20 from *B. subtilis*.

(a) Plasmid R1. Initiation of replication of R1 is dependent on a plasmid-encoded initiator protein, RepA. The minimal region required for RepA-dependent replication (*oriR*) is in-

cluded within a 188-bp DNA region (183) and comprises (i) a 9-bp *dnaA* box, (ii) a contiguous 100-bp region where RepA interacts, and (iii) an adjacent AT-rich region containing three 9-mers. A detailed study of the site of RepA interaction revealed two RepA-binding sites: a preferential RepA site, termed site 1 (5'-CAGTTAAATG-3'), which is adjacent to the *dnaA* box, and a related RepA binding sequence, site 2 (5'-T GTTTAAAAG-3'), for which the protein has a lower affinity. This second site is contiguous to the AT-rich region. Sites 1 and 2 share a core sequence (g/tTTAAA) that is an imperfect palindrome (101). The intervening sequence between the sites shows potential intrinsic curvature. The presence of the *dnaA* box optimizes the action of the DnaA protein at the origin, both *in vivo* and *in vitro*, but it is not absolutely required for the DnaA-dependent replication of R1 (233). EM of replicating intermediates obtained *in vivo* and *in vitro* shows that initiation of R1 replication occurs in a locus that is separated from the minimal origin region (78). A G-type priming signal, located 400 bp downstream of the RepA-binding sequences, has been identified as the site where initiation of the leading strand, primed by DnaG, occurs (186).

(b) Plasmid ColE1. ColE1 is the prototype of a class of small multicopy plasmids that replicate by a theta-type mechanism. Unlike R1, ColE1 does not require a plasmid initiator protein but requires DNA Pol I to initiate replication. The origin of ColE1 replication spans a region of about 1 kb that includes (i) sequences promoting the synthesis of RNA II, the primer of the leading strand (298, 299); (ii) sequences that allow a stable hybridization of RNA II to DNA (139, 189); (iii) sequences that favor specific processing of this coupled complex by RNase H, which generates the 3' end needed to prime leading-strand synthesis (122, 139); (iv) a primosome assembly site (*pas* or *ssi4*) that allows the loading of the DnaB helicase and DnaG primase to initiate the discontinuous priming of the lagging strand (28, 189, 220) (a *dnaA* box that is close to *pas* can be used as a DnaA-dependent DnaB-DnaC assembly site [269, 270]); and (v) a sequence for termination of lagging-strand synthesis, *terH*, which determines unidirectional replication (57, 198). The first two sequences are the most relevant, since they are required for ColE1 replication in the presence or absence of DNA Pol I and RNase H (57, 158, 211). The origin of ColE1 replication, defined as the transition point between RNA II primer and DNA synthesized by DNA Pol I, has been positioned 555 bp downstream of the start point of RNA II (24, 300). This transition point corresponds with data obtained *in vivo* for plasmid pMB1 (closely related to ColE1) (29). Analysis of replication intermediates of ColE1 by EM, located a single origin and showed that replication is unidirectional. At an early stage, leading-strand synthesis proceeds in the absence of lagging-strand synthesis (297, 298).

(c) Plasmid pLS20. An interesting example of plasmid from gram-positive bacteria is the *B. subtilis* plasmid pLS20, for which a preliminary characterization has been reported (192). This plasmid replicates by the theta mechanism, and its replication is independent of DNA Pol I and of a Rep initiator protein. Several palindromes flanking a putative *dnaA* box are located within the origin of replication of pLS20.

Rep proteins. Up to now, dozens of plasmids have been isolated from most bacteria, but not many of them have had their basic replicons dissected and characterized to the level of their nucleotide sequence, and even fewer replicons have been genetically and biochemically studied in detail. The classic way of classifying plasmids is to distribute them among incompatibility groups, whose members have very similar origin sequences and replication control mechanisms. However, due to the difficulty to cope with a complex experimentally based

classification of each newly isolated replicon, a criterion based on sequence comparisons appears to be much more practical. Such a criterion could be the comparison of the amino acid sequences of Rep initiator proteins, since they are encoded by most of the plasmids and they share common functions. Rep proteins recognize specific sequences at the origin of replication, similar to the DnaA initiator protein in bacterial chromosomal replication, and they generate a nucleoprotein initiation complex in which essential macromolecular interactions take place (Rep-DNA, Rep-Rep, and Rep with other initiation proteins of the host) (30). In addition, many Rep proteins can generate complexes that negatively regulate their synthesis and the frequency of initiation.

Based on amino acid sequence alignments of multiple Rep proteins from theta-replicating plasmids, it is possible to construct phylogenetic trees like the one depicted in Fig. 2b. It must be considered that for plasmid sequences, evolution can occur not only by mutation and selection but also by horizontal gene transfer. This constitutes an additional difficulty in establishing evolutionary relationships among plasmids. The phylogenetic tree groups replicons with similar replicative features: plasmids with Rep proteins binding to iterons (like pPS10, pSC101, R6K, and F) cluster apart from others whose initiators bind to nonrepeated sequences (R1 and its relatives), whereas broad-host-range plasmids (RK2, RA1, RSF1010, and TF-FC2) and replicons with dissimilar initiation mechanisms (phage lambda and phasyl) cluster in separated branches. Figure 2a shows an amino acid alignment of a large family of iteron-binding Rep initiators (encircled in Fig. 2b), comprising most of the best-characterized plasmids. The use of such alignments has allowed us the identification of Rep protein motifs, involved in protein-protein interaction (leucine zipper [LZ]) and in DNA binding (α -helix-turn- α -helix [HTH]) (93, 94, 103). A recent *in vitro* study performed with pPS10-RepA has revealed the existence of two globular domains, joined by a flexible linker, in a region of the protein located C-terminal to the LZ motif (102). Protein conformational changes are coupled to the dissociation of RepA dimers (which have a compact package of both domains) into monomers (with an elongated arrangement of the domains). The LZ motif and, to a lesser extent, the first globular domain mediate RepA dimerization. In the compact dimer, the second domain (including the HTH motif) binds to each arm of the operator sequence. In the elongated monomers, the second domain binds to the 3' end of each iteron sequence whereas a DNA-binding activity in the first domain (previously cryptic) is responsible for additional recognition of the 5' half. The sequence alignments in Fig. 2a support a similar structural organization for other Rep proteins of theta-replicating plasmids.

(i) **Protein-protein interactions: the leucine zipper-like motif.** A protein-protein interaction motif resembling the LZ is present in several plasmid-encoded Rep proteins. The LZ motif is responsible for dimerization in several eukaryotic regulatory proteins, through formation of two-stranded coiled coils (172). LZ-like motifs have been detected in the N-terminal region of the Rep proteins of several plasmids (103, 215) (Fig. 3). A mutational analysis has been carried out in the LZ-like motif of the RepA protein of plasmid pPS10 (94). Substitutions of the first two Leu residues of the LZ-like motif (d position according to a coiled-coil nomenclature) with Val resulted in a 13-fold decrease in the RepA association constant (as determined by sedimentation equilibrium analysis of maltose-binding protein-RepA fusions). This finding indicates that the LZ-like motif is a protein-protein interaction interface that regulates the equilibrium between monomers and dimers of the RepA protein. A conservative Ala \rightarrow Val change in a

different residue of the motif (b position) has no effect on monomer-dimer equilibrium, which points to a relevant and specific role in dimerization for the Leu residues of the motif. RepA mutants having Leu \rightarrow Val substitutions were still able to interact *in vitro* with the iterons of the origin, indicating that the LZ-like motif is not directly involved in the binding of RepA to DNA. Further analyses indicated that RepA monomers bind to the iterons of the origin of replication whereas dimers of the protein bind to the *repA* promoter region, pointing to the functional relevance of the two forms of the RepA protein. Similar results have been obtained with the RepA protein of plasmid pSC101. This protein exists in a monomer-dimer equilibrium, although it is mainly in the monomeric form at the protein concentration present in cells harboring wild-type pSC101 (134). However, when the *repA* gene is overexpressed, replication is inhibited (133, 308). Inhibition under overexpression conditions was explained by assuming that elevated concentrations of RepA would promote its dimerization and that the RepA dimers would hinder the interaction of the active RepA monomeric forms with the iterons of the origin (134). Since overproduction of host DnaA protein can reverse inhibition by excess of RepA (133), an alternative explanation to understand inhibition by excess of initiation proteins involves titration of host replication factors.

A region of the pPS10-RepA protein probably involved in protein-protein interactions with host replication factors has been identified. This information was obtained from the analysis of mutations that allowed the establishment of pPS10 (originally a narrow-host-range plasmid from *Pseudomonas savastanoi*) in *E. coli* (85, 104). Three independently isolated mutations were located within the region encoding the LZ motif of RepA. They all resulted in the same Ala \rightarrow Val change (I+5 position; Fig. 3). Other mutations that broaden the host range of pPS10 map in residues adjacent to the LZ-like motif (180), indicating that the RepA region responsible for this phenotype, although partially overlapping, is different from the LZ motif. Since some of the mutations broadened the pPS10 host range without altering RepA-RepA, RepA-*oriV*, or RepA-*repA* promoter interactions (94), it would appear that these changes in the pPS10 initiator should favor proper RepA interactions with host initiation proteins.

Genetic analyses revealed later that the LZ-like motifs found in other Rep proteins play a relevant role. For instance, a mutation that affects the LZ-like region of the R6K initiator protein π resulted in a protein that failed to activate the α or β origins of replication (199). Translation of the gene for the π protein of R6K, starting from an internal initiation codon, can give rise to shorter protein variants in which most of LZ is deleted. This could represent a mechanism for regulation of the level of active replication protein (87). Another example is found in the RepA protein of plasmid pSC101, in which a mutation located in the proximity of the region encoding the LZ-like motif increases the copy number of this plasmid (133).

The existence of protein-protein interfaces apart from the LZ motif in initiator proteins is supported by several lines of evidence. First, the initiator protein of plasmid R1, RepA, interacts cooperatively with DNA sequences at the origin of replication, *oriR* (see above). A mutation located at the 3' end of *repA* results in a thermosensitive replication phenotype (232). The protein variant conserves its ability to interact specifically with *oriR*, but the mutation affects the cooperativity of these interactions (101). This indicates that the mutation has affected a protein-protein interface and suggests that this interface could be located in the C-terminal region of RepA. Mutations affecting RepA residues involved in binding to *oriR* have not been described. Second, a single-amino-acid change

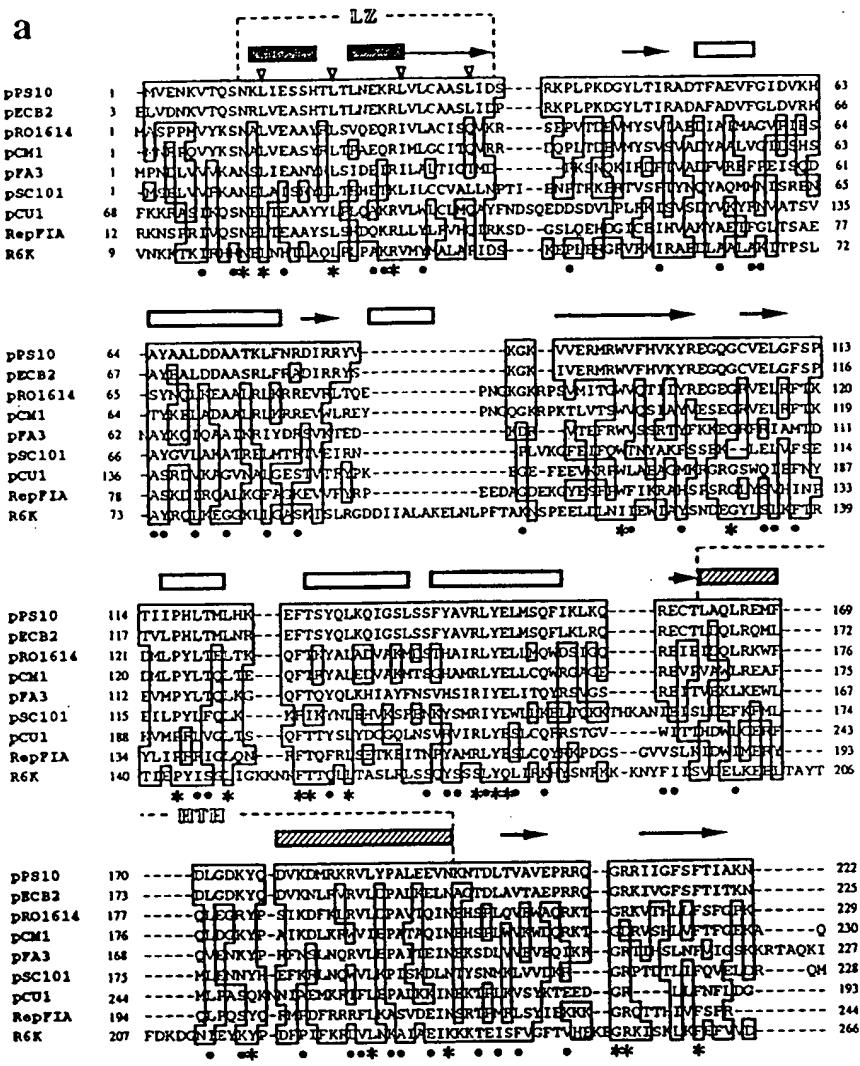


FIG. 2. Sequence alignment and phylogenetic tree of Rep initiator proteins from theta-replicating plasmids. (a) Sequence alignment of the Rep initiator proteins of nine related plasmids of the iteron-containing class. Sequences were aligned with the program CLUSTAL W (version 1.5) by using, for pairwise alignment, gap opening and extension penalties of 10.0 and 0.1, respectively, and the protein weight matrix BLOSUM30. For multiple alignment, the delay for divergent sequences was set to 40% (294). The degree of sequence identity to the pPS10 initiator sequence for conserved residues is shown: *, identical residues in eight or nine of the total sequences; •, identical residues in five to seven sequences. Over the sequences is shown a secondary-structure prediction performed by the neural network algorithm PHD (260) on the output from CLUSTAL W: predicted α -helical regions (boxes) and β -strands (arrows). The two characteristic motifs found in the Rep initiators, LZ (hydrophobic heptad residues pointed to by open arrowheads) and HTH, are indicated. The EMBL database accession numbers are as follows: pPS10, X58896; pECB2, Y10829; PRO1614, L30112; PCM1, X86092; PFA3, M31727; pSC101, K00828; PCU1, Z11775; RepFIA, Y00547; R6K, M65025. (b) Phylogenetic tree for theta-type replicons from gram-negative bacteria, based on sequence alignments of their Rep proteins (such as the one shown in panel a for the pPS10 family, encircled in the tree with a dashed line). The sequences for 35 initiators were retrieved from databases, and a preliminary alignment was performed with CLUSTAL W (data not shown). Sequences that were virtually identical (pairwise scores, ≥ 90) were discarded, and a refined alignment was used as input data for the DISTANCES program of the Genetics Computer Group software package (95). Distance matrices were corrected for multiple substitutions by the method of Kimura. The phylogenetic tree was built up according to the UPGMA method with the program GROWTREE (95). For further discussion, see the text.

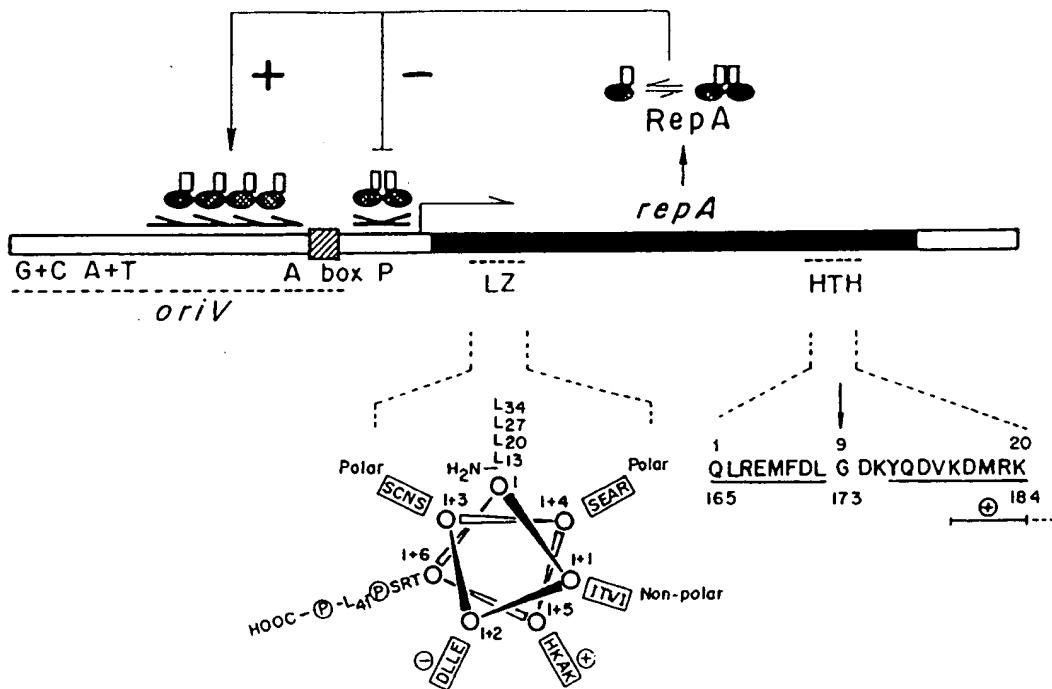


FIG. 3. The theta-type replicon of the *Pseudomonas* plasmid pPS10. The iteron-containing origin (*oriV*) and motifs found in the replication initiator protein (RepA) are depicted. The minimal origin (*oriV*) of pPS10 plasmid is a good example for a "canonical" iteron-containing origin. It is composed of four contiguous and identical 22-bp iterons arranged as direct repeats (half arrows), flanked by a 9-bp *dnaA* box (hatched) and AT- and GC-rich sequences (215). The pPS10 replicon also contains the *repA* gene, encoding the RepA initiator protein (shadowed ovals). RepA is under a monomer-to-dimer equilibrium, which has consequences for protein activity: RepA dimers bind to an inverted repeat (with a sequence partially homologous to that of the iterons) that overlaps with the *repA* promoter (P), acting as self-repressor of *repA* expression, whereas RepA monomers bind to the iterons to form the initiation complex (94). Protein motifs found in RepA are depicted under the protein gene. The LZ motif, responsible for protein dimerization, is represented as a helical wheel projection, in which the hydrophobic spine of Leu residues and the chemical nature of the other displayed residues is indicated (103). For the HTH motif, involved in binding to DNA, the two proposed α -helices are underlined and a stretch of basic residues at the C end of the DNA recognition helix is indicated (+), whereas an arrow points to the Gly residue thought to start the turn (93).

at the N-terminal end of the initiator protein π of plasmid R6K allows this protein to discriminate between palindromic and nonpalindromic binding sites (325). It has been proposed that the change alters a protein-protein interface which modulates interactions of π protein with differently arranged DNA target sequences. Third, RK2 is a broad-host-range plasmid, a characteristic that is related to the existence of two forms of the replication protein, TrfA-44 and TrfA-33. The larger form, TrfA-44, is required for replication in *P. aeruginosa* (80, 274). The shorter version, TrfA-33, starts in an internal initiation codon of *trfA* and promotes the establishment of RK2 in most of its hosts, including *E. coli* and *P. putida*. In addition, the origin requirements are different in the two cases (56, 142, 213, 241). A mutation at the 3' end of the *trfA* gene (affecting the two versions of the protein) modifies the host range of RK2 without altering the binding of the protein to DNA (45, 175; also see reference 241). These results suggest that the C terminus of TrfA plays an important role in the interactions of the initiator protein(s) with host replication factors. Interactions of plasmid initiator proteins with host replication factors have been reported in different systems: (i) the DnaJ protein interacts with the initiation protein of plasmid P1 (312a) and with other chaperones, promoting the efficient binding of this initiator to the origin of replication; (ii) the DnaA protein requires a functional interaction with the RepA protein of plasmid R1 to enter the DnaA box present in the origin of replication (184) (this protein interaction seems to be sufficient to promote DNA replication in the absence of the DnaA box [233]); and (iii) most interestingly, the DnaA, DnaB, and DnaG pro-

teins of the host interact with the π protein of plasmid R6K (16a, 258a) (mutations in the π protein that disrupt the interaction with the DnaA protein are defective in R6K replication [16a]; the specific regions of DnaB and π proteins involved in their interaction have been defined [258a]).

(ii) Specific binding of Rep proteins to DNA: the helix-turn-helix motif. As mentioned above, Rep proteins are able to specifically recognize DNA sequences in the origin region. In addition to this, some of the Rep proteins autoregulate their own synthesis at the transcriptional level by binding to sequences in the *rep* promoter (operator) which show some degree of homology to those present in the origin region. When autoregulation exists, either a single species of the protein is involved in both regulation and replication, or different species of the protein, monomeric and dimeric, recognize the origin and the regulatory regions, respectively (discussed in reference 51). *rep* mutants leading to impaired Rep protein-DNA interactions have been found in various plasmids.

The *Pseudomonas* plasmid pPS10 contains four identical iterons in its origin of replication and an inverted repeat (IR) in the *repA* promoter region. The iterons and IR have partial sequence similarity (92). RepA variants that fail to repress the *repA* promoter had amino acid changes within or in the vicinity of an HTH motif located at the C-terminal end of the protein (93). This motif has been described in many prokaryotic DNA-binding proteins, where it is involved in binding to specific regulatory DNA regions (39, 235). The RepA proteins affected in the HTH motif failed to interact with both the *repA* promoter and the *oriV*, indicating that the motif is involved in

interactions with both the DNA regions. A working model proposes that the RepA protein contacts the inverted repeats of the *repA* promoter region as a dimer, using the HTH motif (92, 93). This HTH motif also binds to a conserved 3' region in the iterons, which in their 5' ends would be bound by another region of the RepA protein (102). A similar model has been postulated for other plasmid Rep proteins, in which monomeric and dimeric forms of the protein are involved in replication and autoregulation, respectively (discussed in reference 51).

In plasmid RK2, mutations that lead to TrfA protein variants affected in binding to the origin were scattered over a *trfA* gene region encoding the 162-amino-acid C-terminal moiety of the protein (46). In plasmid pSC101, the last third of the RepA protein is not needed for binding to specific DNA sites (181), which contrasts with the role of the C-terminal region in other initiators.

Initiation and elongation of replication. (i) **DNA replication dependent on plasmid initiators.** Initiation of replication requires the assembly of the complete replication machinery including DNA polymerase III holoenzyme (DNA Pol III-HE), DnaB helicase, and primase at the plasmid origin. Once the checkpoint corresponding to the initiation of leading-strand synthesis is past, replication continues until completion, following a process catalyzed by DNA Pol III and other host proteins. Most of the theta-type replicons require, at least, a plasmid-encoded Rep protein and the host DnaA protein for the initiation step. The general organization of the origin region in these plasmids resembles the arrangement found in *oriC* (30). The plasmid *ori* includes not only the specific sequences where the Rep and DnaA proteins interact but also an AT-rich region containing direct repeats, analogous to the 13-mers in *oriC*, where the DNA strands are melted. The AT-rich repeats have also been involved in the transfer of the DnaB-DnaC complex to *oriC*. In the theta-replicating plasmids, the Rep protein binds to specific sequences in the origin, forming a nucleoprotein preinitiation complex analogous to the one formed by DnaA at *oriC*. The Rep-DNA complex, in combination with DnaA, facilitates the transfer of the DnaB-DnaC complex to the origin and the opening of the strands in the AT-rich region. The structural organization of the initiation complex could be facilitated by host factors such as HU, IHF, or FIS. The assembly of the preinitiation complex and details of the molecular interactions leading to the initiation of replication are well documented for a few theta-replicating plasmids (described below).

(a) **Plasmid pSC101.** RepA, the initiator protein of plasmid pSC101, exists in a monomer-dimer equilibrium, which determines the efficiency of RepA in replication (134). Monomers and dimers of RepA are both functional, but they play different roles: monomers bind to the iterons at the origin, promoting initiation, whereas dimers bind to the adjacent inverted repeat, repressing transcription of the *repA* gene (181). However, interactions of the RepA dimers with the inverted repeat also play a role in replication in the absence of the *par* locus (197). Initiation of pSC101 replication requires, in addition to RepA (308), the DnaA host replication initiator (113), and IHF proteins (91, 283). Binding of IHF to its target, within the AT-rich region, leads to DNA bending (283), which promotes interactions between DnaA molecules bound to *dnaA* boxes separated by some 200 bp (282). Binding of RepA to the origin region further stabilizes DnaA contacts with the distant *dnaA* boxes (282). The RepA-DNA-DnaA complex plays a role in replication but also in partitioning of the plasmid (54). Stable plasmid inheritance requires the *par* locus, which is close to the origin region: this locus contains a site for DNA topoisomerase

II and also determines the proper supercoiling at the origin region needed for initiation (53, 132).

(b) **Plasmid P1.** Plasmid P1 replication is dependent, both in vivo and in vitro, on the specific initiator protein RepA (6, 313) and on the host DnaA protein (110, 313). Formation of the initiation complex requires the monomeric form of RepA (315), and RepA-DNA binding is stimulated by heat shock chaperones. The latter proteins could contribute to the dissociation of the RepA dimers into monomers, which is the form of the RepA protein that recognizes the five iterons of the origin (58, 315). However, growing evidence indicates that the chaperones are required to activate the monomers of RepA (50, 78a, 236). Binding of the activated RepA monomers to the five iterons of the origin results in wrapping of the DNA around RepA, presumably due to in-phase bending of DNA (206). RepA monomers contact each iteron through two consecutive major grooves on the same face of the DNA helix (242). RepA alone is unable to melt the origin; this role is performed or favored by DnaA, which also stimulates the DNA-binding activity of RepA (206). There is a set of two tandem *dnaA* boxes at one end and a set of three tandem *dnaA* boxes at the other end of the P1 origin. Although either of the sets, or even just one *dnaA* box that conforms exactly to the consensus, is sufficient to support DnaA-dependent replication (4, 36, 38), melting of the origin region by DnaA is maximally efficient when both sets are present, probably due to DNA looping mediated by DnaA bound to the two sets (206). The orientation of the *dnaA* boxes and the different sensitivity of the two strands to reagents specific for single-stranded DNA suggest that DnaA-dependent loading of DnaB preferentially occurs in one of the strands, which can account for the unidirectional mode of P1 replication (206). Efficient replication of P1 requires adenine methylation of the five GATC sites of the origin. These GATC sites are clustered in direct heptamer repeats which are separated from the RepA-binding site by a GC-rich spacer (1, 2, 37).

(c) **Plasmid RK2.** Important information on the initial events of replication of plasmid RK2 has been obtained (161a). The ClpX chaperone yields monomers of the plasmid initiation protein, TrfA, which is the form that is active in binding to the five 17-bp iterons of the origin (161b). This binding promotes, in the presence of HU protein, local strand opening within the AT-rich region of the origin. Interactions of the DnaA protein of the host with four DnaA boxes present in this region are also required for initiation of plasmid replication. These interactions increase, but are not strictly required for, the opening of the strands. DnaA is required for the delivery of the DnaB helicase to the origin region and both DnaA and TrfA are required for DnaB-induced template unwinding. This suggests a role of TrfA in the repositioning and activation of the DnaB activity (79b). The requirement of particular DnaA boxes is host-dependent (79a). This is consistent with the plasticity of the RK2 origin with respect to structural requirements for replication in different bacterial hosts.

(d) **Plasmid R6K.** As stated above, replication from the γ *ori* of R6K requires π protein (96, 160, 272, 278). This protein can recognize different types of DNA sequences: iterons, enhancer, inverted repeats in the promoter of the *pir* gene (encoding the π protein), and the AT-rich segment of the origin (174). The π initiator promotes the initiation of replication from three origins of replication: α , β , and γ (reviewed in reference 87). *ori*- γ is in a central position, separated by 3 and 1.2 kb, respectively, from *ori*- α and *ori*- β . *ori*- γ contains seven 22-bp iterons, flanked by two IHF-binding sites and two *dnaA* boxes. Contiguous to the iterons is an AT-rich region which contains one of the *dnaA* boxes and one of the IHF-binding sites (62).

ori- β contains half an iteron, and *ori*- α one complete iteron that are essential for function. Under standard conditions, *ori*- α and *ori*- β are more active than *ori*- γ , but they depend on a distant enhancer for activity (146, 147). This enhancer partially overlaps *ori*- γ , but its activity and the origin function have been distinguished by mutational analysis.

A dimer of π seems to bind to each of the seven 22-bp iterons present in *ori*- γ (86). Protein π binds preferentially to one of the strands of the *ori*- γ (98) and bends the DNA, generating a wrapped nucleoprotein structure (205). DnaA protein is required for replication from this origin, and it can bind the two *dnaA* boxes that flank the iterons (146, 147). Although two IHF-binding sites are flanking the iterons of *ori*- γ , the preferential or unique binding site(s) is the one located within the AT-rich region (62, 145a). IHF protein binding to these sequences induces conformational changes that are important in the regulation of replication initiation (145a). This binding could also affect the interaction of the π protein with the DnaA initiation protein of the host (16a). In the presence of normal levels of π , IHF is required for replication from *ori*- γ (61). An active *ori*- γ requires the binding sites for π , DnaA, and IHF proteins in the correct geometrical alignment (147). Protein π binds efficiently to the iterons of the *ori*- γ but not to *ori*- β or *ori*- α . However, the enhancer favors the long-range activation of *ori*- β and *ori*- α by transfer of the initiator protein, and possibly other initiation factors, from *ori*- γ (199, 203, 204). The activation of *ori*- β , unlike *ori*- γ and *ori*- α , does not require DnaA protein (147). Three new R6K gene products that distort essential sequences of *ori*- α and *ori*- β have been described (89). However, these proteins have been identified as proteins needed for conjugative transfer rather than for plasmid replication (230a).

(e) *Plasmid R1*. Plasmid R1 is the most extensively studied member of the IncFII family of plasmids. In vivo and in vitro replication of R1 requires the initiator protein, RepA (77, 159, 183, 305). *oriR*, defined as the minimal region required for RepA-dependent replication of R1 in vitro, is bound specifically by RepA (101, 183, 184). Unlike the above cases, *oriR* does not contain iterons. RepA protein, probably as a dimer, recognizes sequentially (albeit with different affinities) the cores of two partially palindromic sequences (Fig. 4) (101). These sequences are located on the same face of the DNA helix, within a region of potential curvature, and are 8 helical turns apart (79). Interactions between RepA molecules bound to each of the sites could be responsible for DNA looping at the ends of a 100-bp region within *oriR* that is protected by RepA against DNase I cleavage (101). Following formation of the initial complex, additional RepA molecules could bind to the intermediate region by cooperative protein-protein interactions, generating a high-order complex. These interactions are needed for replication, as indicated by the defective replication phenotype associated with a *repA* mutant that failed to generate high-order RepA-*oriR* complexes (101, 232). The RepA-DNA complex seems to melt the DNA strands in the AT-rich region (185). In vitro replication of R1 requires DnaA protein (184, 231). DnaA binds to a *dnaA* box that is adjacent to the RepA-binding region, but this binding does not occur, or is very inefficient, in the absence of RepA (184, 233). It is likely that RepA-DnaA contacts guide the entrance of the DnaA protein in *oriR*, because the *dnaA* box is not absolutely necessary for the DnaA-dependent replication of R1 (233). Surprisingly, although in vitro replication of R1 is dependent on DnaA, this protein is dispensable for the replication of IncFII plasmids in vivo (20, 290). In vivo replication in the absence of DnaA is inefficient, but plasmid copy mutants that increase the levels of RepA protein improve the efficiency of replication

(20). These results show the essential role of RepA in origin activation and imply that RepA could promote melting of the DNA strands at the origin and loading of the DnaB-DnaC complexes.

Interactions of RepA protein with the *oriR* region promote, both in vitro and in vivo, initiation of leading-strand synthesis at a DnaG-priming site (the G site, resembling the bacteriophage G4 origin for complementary strand synthesis) (21, 186) that is located 400 bp downstream of *oriR*. It has been proposed that the G site is activated when synthesis of the lagging strand, which initiates at the AT-rich region, reaches the G4-like site. Initiation at the G site, promoted by DnaG, cannot progress toward the origin. This leaves a gap that is filled later in the replication cycle (186). The relevant role played by DnaG in initiation of R1 replication is consistent with the complete inhibition of the in vitro replication of the plasmid by anti-DnaG antibodies (231). Antibodies against the *E. coli* single-stranded DNA-binding protein (SSB) also block in vitro R1 replication (231), indicating the essential role of this protein at an early stage in R1 replication. Finally, it has been established, both in vivo and in vitro, that replication of R1 requires DnaK protein (105). RepA protein of R1 seems to interact initially with the two partially palindromic sequences at the *oriR* region as a dimer, but further binding to the intermediate region could be by monomers, since there are no symmetrical sequences (102). This could support the notion that DnaK plays a role in the activation of R1 replication similar to that proposed for P1 replication (58, 315).

(f) *Plasmids ColE2 and ColE3*. As mentioned above, the smallest of all the prokaryotic origins described so far have been defined in the ColE2 and ColE3 replicons (322). These plasmids require for replication a plasmid-specific initiator, but, like ColE1 (see below), they also require DNA Pol I to initiate leading-strand synthesis. Initiation of ColE2 and ColE3 replication is dependent on the synthesis of specific primer by their Rep proteins (288). A single-strand initiation site (*ssi*) for the priming of DNA replication has been located near the *ori* of ColE2 (219).

(g) *Plasmids of the pAMβ1 family*. Replication of the pAMβ1/pIP501 broad-host-range plasmid family from gram-positive bacteria (32, 41, 42) requires, like ColE2 and ColE3, DNA Pol I and Rep protein. A model based on the synthesis of a primer RNA catalyzed by the host RNA polymerase (RNAP), the specific cleavage of the primer at the origin region (perhaps mediated by the Rep protein), and the extension of the 3' end catalyzed by DNA Pol I has been proposed (41).

(ii) *Replication independent of plasmid-encoded initiator proteins*. The best-characterized replicon that is independent of plasmid-encoded initiator proteins is ColE1. Initiation of ColE1 replication involves the consecutive activities of RNAP, RNase H, DNA Pol I, and DNA Pol III-HE (reviewed in references 163, 182, 248, 280, and 301). Transcription mediated by the host RNAP is required to synthesize the preprimer for leading-strand synthesis. Specific cleavage by RNase H of the preprimer (termed RNA II) annealed to DNA provides a 3' end that defines the starting point for leading-strand synthesis. This synthesis is initially carried out by DNA Pol I. Steric hindrance of the bulky DNA Pol III-HE by the folded RNA II (upstream of the RNA-to-DNA transition point) probably prevents extension of the primer by this polymerase (188). DNA Pol I synthesizes about 400 nucleotides of the leading strand, exposing, on the displaced strand, a primosome assembly site (*pas*). Once the primosome is assembled at the *pas* site, it translocates in the 5' → 3' direction, unwinding the helix and priming the discontinuous DNA synthesis. At this point, DNA Pol I is replaced by the highly processive DNA Pol

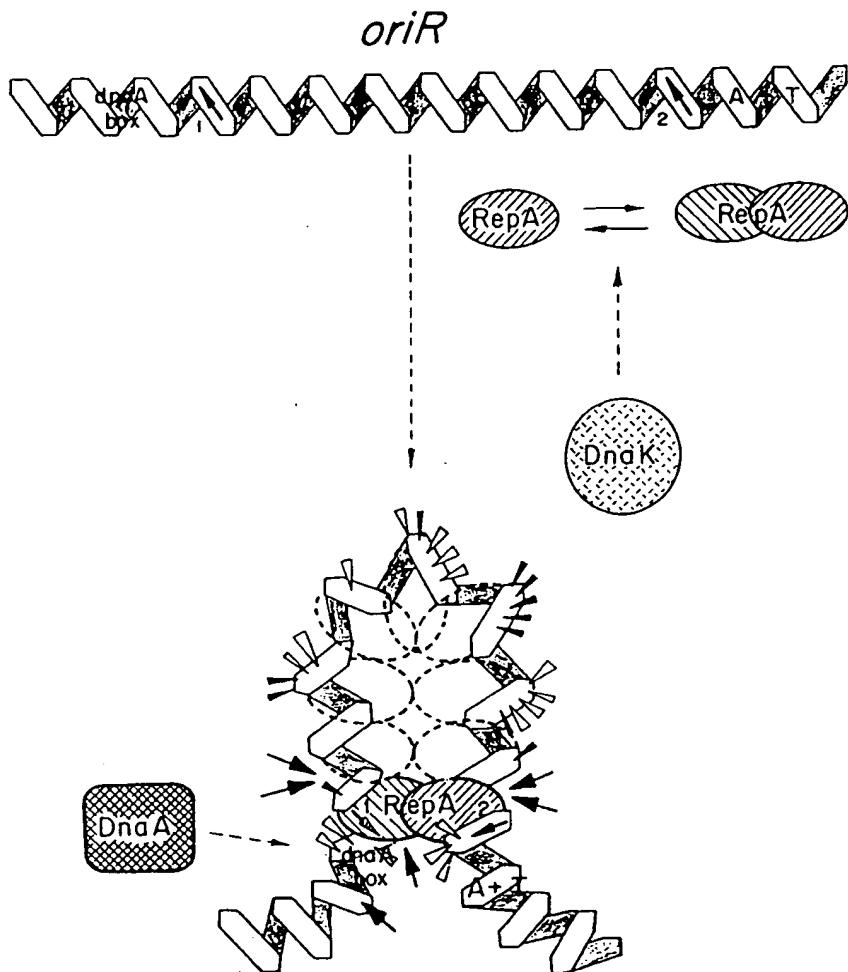


FIG. 4. RepA-*oriR* complexes in initiation of R1 plasmid replication. A 100-bp region in the *oriR* replication origin is continuously bound by the plasmid RepA protein to form the initiation complex. There are no ictrons in *oriR*, but two partially palindromic 10-bp sequences (sites 1 and 2) are found at the ends of that 100-bp region. They are flanked, respectively, by a consensus *dnaA* box and by three AT-rich sequences. These three sequences are believed to be melted to allow the DnaBC complex access to the open origin. A RepA initiator (hatched oval) dimer binds with high affinity to site 1, and then, in a second binding event, a different RepA dimer would bind with lower affinity to the distal site 2 sequence. The DNA of the *oriR* region could be bent to facilitate the topological proximity of sites 1 and 2, which are disposed on the same face of DNA double helix. Binding of RepA dimers to sites 1 and 2 would generate a small DNA loop, held together by protein-protein interactions. The DNA loop would be filled afterwards with more RepA molecules that are brought to the complex mainly by protein-protein interactions. This model is based on experimental gel mobility shift assays and footprinting data with both wild-type and mutant RepA proteins and *oriR* sequences (101). Arrowheads indicate DNase I-hypersensitive sites (the size is proportional to the intensity of cleavage), whereas arrows point to strong cleavage sites for hydroxyl radicals. The interaction of DnaA host initiator with its DNA-binding site is dependent on the previous formation of the RepA-*oriR* nucleoprotein complex (233). A requirement for a DnaK chaperone has been described for R1 DNA replication (105). A hypothetical role for DnaK in modulating the aggregation and activation state of RepA dimers, inspired by the findings for P1 plasmid RepA initiator (316), is also shown.

III-HE. The switch between DNA Pol I and DNA Pol III-HE could be favored by helix-destabilizing proteins bound to the template of the leading strand, which has to be exposed by the DnaB helicase (discussed in reference 280). Leading-strand synthesis can occur uncoupled from lagging-strand synthesis, and DnaG, but not DnaB, is dispensable for this uncoupled synthesis (281). Lagging-strand synthesis, initiated at the *pas* site, extends toward the promoter of RNA II but is arrested at a site (*terH*) 17 bp upstream of the leading-strand initiation site (57). The mechanism of this arrest is unknown. These events determine the unidirectional pattern of ColE1 replication.

Termination of replication. The points at which theta-type replication terminates can be actively determined by molecular interactions at particular sequences. The first replication arrest sequence, *ter*, was identified in plasmid R6K as a barrier to the

unidirectional replication initiated in either *ori*- α or *ori*- β of this plasmid. Replication starts then from the initial origin in the opposite direction and progresses to completion (177). The R6K terminus acts as a temporal barrier to replication initiated in other replicons (160). The nucleotide sequence of the *ter* region was determined, and the replication terminus of R6K was cloned (17, 18). The organization of this sequence as two separable and polar terminus sites was recognized and verified (130). The recognition of the essential features of the *ter* site allowed the identification of similar sites in plasmids of the IncFII (R1 and R100) and IncFI (repFIC) groups, as well as in the chromosome of *E. coli*. The *ter* sequence is the binding site of Tus, a monomeric protein of *E. coli* that promotes the termination of plasmid replication (121, 275). The identification of *ter* homologous sites in the chromosome of *E. coli*

triggered replication termination studies in this bacteria and also in *B. subtilis*, where DNA-arresting sequences (IR-I and IR-II) and a dimeric protein that promotes the termination of DNA replication, RTP, have been identified (reviewed in references 14, 19, and 120). Unlike Tus, which acts as a monomer, RTP acts as a tetramer of two dimers (261, 262).

A major step in understanding active termination of DNA replication was the determination of the three-dimensional structure of the RTP dimer at the atomic level by crystallographic methods (43). This determination allowed the identification of protein-protein interfaces in RTP and opened the way to comparative structural analyses which suggested that RTP folding is similar to the "winged-helix" domain found in a family of DNA-binding proteins (43, 285). The polar arrest of the replication fork caused by RTP protein has been proposed to be due to specific interactions between the terminator protein and the DnaB helicase (116, 151, 172a, 261). Like the *B. subtilis* RTP-IR complex, the *E. coli* Tus-ter complex interferes with the helicase activity of the replisome complex in an orientation-dependent manner (151). Following the determination of the RTP structure, the structure of the Tus-ter complex was also solved by X-ray crystallography (143). This structure provided information on the singular architecture of the Tus protein and on the Tus-ter protein-DNA interactions involved. In addition, these studies support the speculation that the polar arrest of the replication fork, occurring at the Tus-ter complex, could be due to the polar inaccessibility of the helicase to the protein DNA-binding site. The termination of DNA replication is a regulated process, as indicated by the identification of a small protein of *E. coli* that binds to a terminator site and prevents replication fork arrest mediated by the Tus protein (214).

Sequences arresting lagging-strand synthesis, called *terH*, have been found upstream of and close to the *pas* site of ColE1; the arrest seems to be caused by the nonhybridized portion of RNA II (57, 212). It has been found that in multimers containing ColE1-type replicons oriented head-to-head, one origin of replication acts as a polar pausing site for replication initiated in the other origin (307). It is possible that this pausing is due to the stalling of the replication fork by the unhybridized portion of RNA II. Alternatively, the replisome could be transiently stalled at a protein-DNA complex, such as the *pas* site (discussed in reference 307). The potential role of an initiator protein to arrest replication progressing toward the origin has been reported in plasmid R1 (168). Active stalling of replication forks could be important for determining the direction of replication and for accurate termination and may modulate the efficiency of replication or the coupling between replication and cell division.

During the final stages of plasmid replication, catenates containing gaps in both daughter strands can be originated (212). These catenates can be resolved by either type I or type II topoisomerases. Genetic analysis revealed the involvement of a specific type II topoisomerase, Topo IV, in the segregation of plasmids and bacterial nucleoids (145). A two-stage model for the segregation of the replication products has been proposed (8, 9), with DNA gyrase reducing the linking number during elongation of DNA synthesis (stage I) and Topo IV resolving the supercoiled catenates which are the products of replication (stage II). Although cross-activities of DNA gyrase in decatenation and of Topo IV in supporting fork progression can be detected, *in vivo* and *in vitro* data confirm the specialized role of Topo IV in unlinking daughter replicons (117, 245, 246, 327). Maturation of the open-circular forms arising from the decatenation by Topo IV into supercoiled molecules can be efficiently carried out by DNA gyrase. Data obtained with

plasmid R1 indicate that maturation of newly replicated DNA molecules is a slow process, which prevents rapid reutilization of the last replicated molecules (221). As the result of an odd number of homologous recombination events, dimers can arise. Replication intermediates can provide ideal substrates for these homologous recombination events. The resolution of DNA dimers by specialized systems can also be considered part of the replication termination process (discussed in reference 19).

Synopsis. Replication by the theta-type mechanism is widespread among plasmids from gram-negative bacteria and has also been reported in plasmids from gram-positive bacteria. EM shows that replicating intermediates appear as bubbles (early stages) that, when they increase in size, result in theta-shaped molecules. Two early events in this mode of replication are the opening of the strands at specific sequences (the origin of replication) and the synthesis of RNA primers. Opening of the strands is catalyzed by specific initiators (Rep and DnaA proteins) and/or by transcription by RNAP. Initiation proteins promote, at the origin of replication, the sequential assembly of components of the replisome complex. The main replicative helicase of the cell catalyzes further unwinding of the strands. RNA primers are synthesized either by RNAP or by bacterial or plasmid primases. DNA synthesis of both strands is coupled and occurs continuously on one of them (leading strand) and discontinuously on the other (lagging strand). DNA Pol III is required for elongation of plasmid DNA replication. In addition, DNA Pol I can participate in the early synthesis of the leading strand (ColE1 and pAMβ1). Theta-type replication is, in most cases, unidirectional. Topoisomers are originated at termination (right-handed catenates), and their resolution requires the participation of Topo IV. Termination of DNA replication is determined in some plasmids by specific protein-DNA complexes.

Strand Displacement Replication

The best-known examples of plasmids replicating by the strand displacement mechanism are the promiscuous plasmids of the IncQ family, whose prototype is RSF1010. Members of this family require three plasmid-encoded proteins for initiation of DNA replication. These proteins promote initiation at a complex origin region, and replication then proceeds in either direction by a strand displacement mechanism (266; reviewed in reference 263).

Origins of replication. The origin of replication of plasmid RSF1010 has been defined as the minimal region able to support bidirectional replication when the RSF1010 replication proteins (RepA, RepB, and RepC) are supplied in *trans* by a second plasmid (266). This region also contains the origin of replication, as defined by EM analysis of replication intermediates obtained *in vivo* (59) and *in vitro* (266). The minimal *ori* region includes three identical 20-bp iterons plus a 174-bp region that contains a GC-rich stretch (28 bp) and an AT-rich segment (31 bp). The origin extends further with a nonessential region and two small palindromic sequences containing the *ssiA* and *ssiB* sites located in opposite strands (Fig. 5a). Iterons are the RepC-binding sites (111, 112). The inverted repeats at the *ssi* sites could favor the formation of hairpins. In these hairpins, base complementarity in the upper part of the putative stem is essential for replication, while base complementarity and sequence specificity in the lower part of the stem are important for primer synthesis (195). The *ssiA* and *ssiB* sequences are specifically recognized by the plasmid-encoded RepB primase, which primes continuous replication from these sequences (111, 128, 129). Genetic analysis indicated that

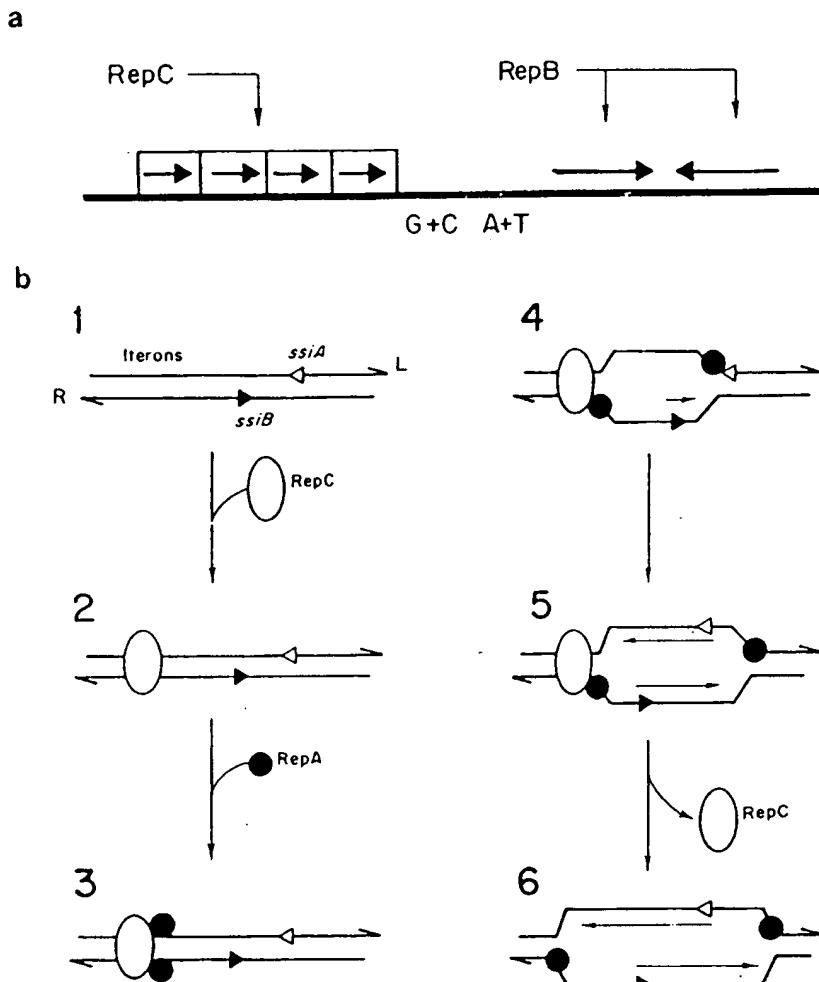


FIG. 5. Replication of plasmid RSF1010 by the strand displacement mechanism. (a) Origin of replication and related regions. The interaction sites of RepB (inverted repeat [convergent arrows]) and of RepC (iterons [boxed arrows]) are indicated. GC- and AT-rich regions are also depicted. (b) Model for initiation of replication by the strand displacement mechanism in plasmid RSF1010 (266). Replication occurs with opposite polarities from two origins (*ssiA* and *ssiB*), which are independently used. Interactions between the plasmid-encoded proteins RepC and RepA are indicated. Priming is catalyzed by RepB' (not shown). Thin lines indicate newly synthesized DNA, with the direction of synthesis indicated by arrowheads. See the text for details.

a single *ssi*, in an orientation that favors priming and chain elongation away from the iterons, is sufficient for RSF1010 replication (118). This organization suggests that the origin of replication of RSF1010 can be separated into three functional loci: the iterons, the *ssiA* region and the *ssiB* region. The iterons and the adjacent AT-rich region function as a duplex-opening region, and the *ssiA* and *ssiB* sites form a priming region (263).

Rep proteins. As indicated above, replication of RSF1010 is promoted by the joint activity of three plasmid-encoded proteins, RepA, RepB, and RepC, that have, respectively, 5'-3' helicase, primase, and initiator activity (111, 263). The RepC protein, a dimer of 31-kDa subunits, interacts specifically with the iterons of the origin (111, 112) and probably with the RepA helicase, promoting the exposure of the *ssi* sites in a single-stranded DNA (ssDNA) configuration (129, 266, 289). The RepA protein is a hexamer of 30-kDa subunits, and it contains two activities: an ssDNA-dependent ATPase and a 5'-3' DNA helicase. Expression of *repB* from two in-frame alternative start codons results in two polypeptides of 36 and 38 kDa,

which correspond to two functional forms of the RepB primase: RepB and RepB' (111, 267). The 38-kDa RepB' was shown to be identical to the RSF1010-encoded MobA protein (involved in conjugative mobilization) (266).

Replication mechanism. Replication of RSF1010 DNA is independent of the host-encoded DnaA, DnaB, DnaC, and DnaG proteins, whose roles are played by the combined action of the plasmid-encoded RepA, RepB, and RepC proteins (90, 111, 265). The template for initiation of RSF1010 replication is supercoiled plasmid DNA (78, 266). DNA Pol III-HE and SSB are required for replication. Figure 5b outlines a model for initiation of RSF1010 replication, proposed by Scherzinger et al. (266). The first stage of this process involves the binding of the RepC protein to the iterons of the origin. It is assumed that the RepA helicase binds to both DNA strands in the AT-rich region, close to the site of interaction of RepC. Subsequent translocation in the 5'-3' direction of the RepA helicase bound to the L strand (the DNA strand which has the same sequence as the mRNAs coding for 10 of the 11 known RSF1010 proteins) (267) melts the duplex, exposing and acti-

vating the *ssi* sites. Alternatively, the interaction of RepC with the iterons could induce the opening of the duplex near the *ssi* sites. The exposure of the stem-loop structure in the *ssi* sites is probably required for the assembly of the RepB-primase to initiate replication (195). Initiation at either *ssi* site can occur independently, and replication proceeds continuously, with the RepA helicase facilitating displacement of the nonreplicated parental strand as a D loop. Continuous replication from each *ssi* signal in opposite directions would originate a double-stranded DNA theta-shaped structure in the overlapping region and two D loops beyond this region. The helicase activity of the RepA protein is required during the elongation of RSF1010 replication, and this protein cannot be replaced by the host DnaB helicase. The RepA helicase of RSF1010 works in the 5'→3' direction, which implies that it is working while bound to the displaced strand. The end products of the strand-displacement replication mechanism are ss-displaced circles and double-stranded supercoiled circles. The ssDNA molecules could correspond to either DNA strand and therefore could contain either the *ssiA* or *ssiB* sequences. These sequences are used to initiate synthesis of the complementary strand, which converts the ssDNA templates into double-stranded supercoiled circles. Therefore, double-stranded DNA (dsDNA) molecules, displaced single-stranded circular molecules, and partial double-stranded circles can be formed in this mode of replication.

Synopsis. IncQ plasmids (typically RSF1010) are replicons that can be propagated in many different hosts. Replication of RSF1010 occurs from two symmetrical and adjacent single-stranded origins (*ssiA* and *ssiB*) positioned one on each DNA strand. Replication starts when these origins are exposed as single-stranded regions. The melting of the DNA strand is dependent on two plasmid replication proteins, RepC and RepA, and is facilitated by an AT-rich region that precedes the *ssiA* and *ssiB* regions. RepC recognizes directly repeated sequences of the origin adjacent to the AT-rich region, and RepA is a DNA helicase. Priming of DNA synthesis at these origins is catalyzed by the plasmid-specific primase (RepB). Synthesis of each one of the strands occurs continuously and results in the displacement of the complementary strand. Replication of this displaced strand is initiated at the exposed *ssi* origin. Due to the activities of the three plasmid replication proteins (RepA, RepB, and RepC), initiation of RSF1010 replication is independent of transcription by host RNAP and of host replication factors acting at the early replication stages (DnaA, DnaB, DnaC, and DnaG). This independence may account for the broad-host-range character of the IncQ replicons.

Rolling-Circle Replication

Replication by the RC mechanism has to be unidirectional, and it is considered to be an asymmetric process because synthesis of the leading strand and synthesis of the lagging strand are uncoupled (reviewed in references 69, 84, 108, 150, 150a, and 228). One of the most relevant features of RC replication is that the newly synthesized leading plus strand remains covalently bound to the same parental plus strand. RC replication was originally thought to be limited to ssDNA coliphages and to small multicopy plasmids isolated from gram-positive bacteria. However, there are known instances of plasmids isolated from gram-negative bacteria, from cyanobacteria, and from species of *Archaea* that use the RC mode for replication (83, 99, 127, 156, 324, 331). Although most of the RC-replicating plasmids so far described are smaller than 10 kb, all small plasmids do not necessarily replicate by the RC mode.

For example, small plasmids like pRJF1 (2.6 kb) and pWV02 (3.8 kb), isolated from gram-positive bacteria, replicate by the theta mode (115, 152). Studies on the molecular mechanisms underlying RC replication have been done mainly with the staphylococcal plasmids pT181 (228), pC221 (292), pUB110, and pC194 (108) and with the streptococcal plasmid pMV158 and its *Δmob* derivative pLS1 (69).

The current model for RC replication involves several experimentally distinguishable stages (Fig. 6). Replication is initiated by the plasmid-encoded Rep protein, which introduces a site-specific nick on the plus strand, at a region termed double-stranded origin (*dso*). The nick leaves a 3'-OH end that is used as a primer for leading-strand synthesis, which most probably involves host replication proteins (at least DNA Pol III, SSB, and a helicase). Elongation from the 3'-OH end, accompanied by the displacement of the parental plus strand, continues until the replisome reaches the reconstituted *dso*, where a DNA strand transfer reaction(s) takes place to terminate leading-strand replication (see below). Thus, the end products of leading-strand replication are a dsDNA molecule constituted by the parental minus strand and the newly synthesized plus strand, and a ssDNA intermediate which corresponds to the parental plus strand. Unlike replication by the strand displacement mechanism, the ssDNA intermediates generated by the RC replication mode correspond to only one of the plasmid DNA strands. The pioneering work in Ehrlich's laboratory showed that generation of ssDNA is the hallmark of plasmids replicating by the RC mechanism (108, 291). Finally, the parental plus strand is converted into dsDNA forms by host proteins initiating at the single-strand origin (*sso*), which is physically distant from the *dso*. The last step would be the supercoiling of the replication products by the host DNA gyrase.

Origins of leading-strand synthesis. RC-replicating plasmids are made up of interchangeable gene modules (253). There is only one essential module. This harbors the functions for plasmid replication and includes the *dso*, the *rep* gene, and the plasmid elements involved in replication control. This module has been named the leading-strand initiation and control (LIC) region (69). In addition, plasmids may include an antibiotic resistance determinant, a gene involved in conjugative mobilization (*mob*), and one or two *sso* regions. Based on the homologies observed in the essential LIC module, four main plasmid families have been defined, their prototypes being pT181, pC194, pMV158, and the staphylococcal plasmid pSN2 (69, 84, 108, 228). Little information is available on the fourth plasmid family, and newly described plasmids from *Pyrococcus abyssi* and from extreme halophiles seem to belong to one of these families or to fall within a different, less well characterized, fifth family (83, 127). An interesting replicon is the natural phage-plasmid hybrid replicon, termed phasyl, which has been isolated from *E. coli*. It contains two functional origins: one has homology to the viral and complementary origins of the ssDNA coliphage M13, whereas the other origin requires activation by the phasyl-encoded Arp protein. Iterons or *dnaA* boxes typical of the theta-type replicons have not been found in phasyl (271).

Two loci have been defined within the *dso*, namely, the *bind* and *nic* regions (70). The former locus includes the sequences needed for the Rep protein to bind to the plasmid DNA, whereas the latter contains the site where the Rep protein introduces the initial nick (Fig. 7). The two loci can be contiguous (pT181 family) or can be separated by up to about 100 bp (pMV158 family). A typical feature of the RC-replicating plasmids is that the *nic* regions are highly conserved among replicons of the same family whereas differences are found at the

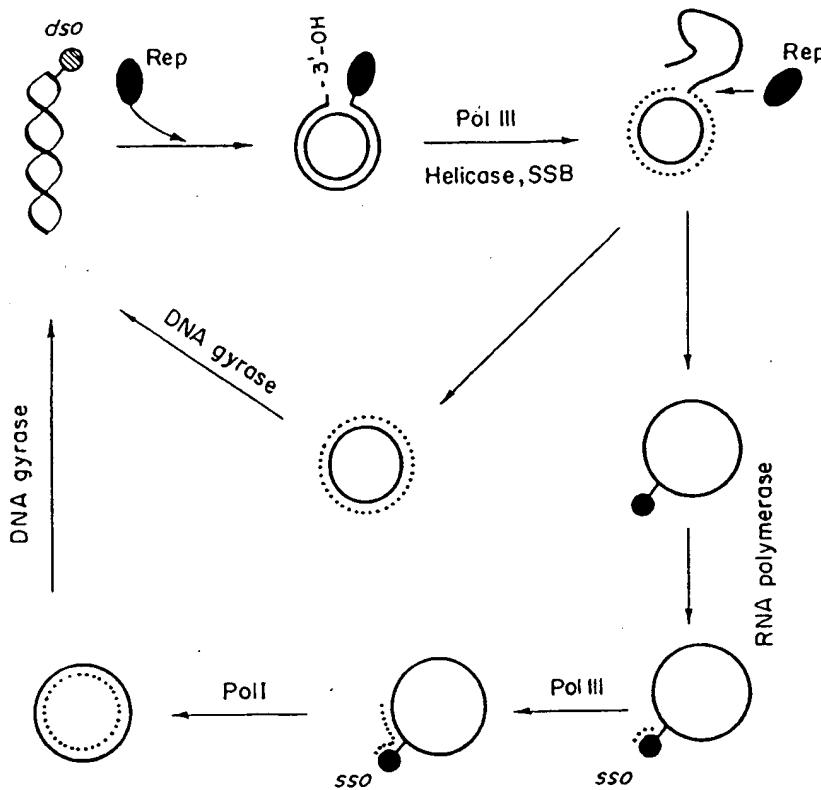


FIG. 6. Model for RC replication. The plasmid-encoded Rep protein recognizes the *dso* on supercoiled DNA and introduces a site-specific nick generating a free 3'-OH end. This end is elongated by host proteins as the parental strand is being displaced. When the replication fork reaches the reconstituted *dso*, Rep protein catalyzes a strand transfer reaction, releasing an ssDNA intermediate and a dsDNA molecule with a parental and a newly synthesized (dotted) strand. Lagging-strand synthesis on the ssDNA molecule is initiated at the *sso* signal by the host RNA polymerase. This enzyme would synthesize a short primer RNA, and lagging-strand synthesis is performed by host DNA polymerases. The end products are supercoiled plasmid DNA molecules.

bind loci. This fact shows that Rep proteins of plasmids from the same family must have a common catalytic domain for phosphodiester bond cleavage and sealing whereas replicon specificity (i.e., Rep binding to the *bind* region) would be located in a different, nonconserved domain (see below). The DNA sequences of the *bind* regions are either an inverted repeat contiguous to the nick site (IR-III in the pT181 and pC194 families [Fig. 7]) or a set of two or three direct repeats separated from the nick site by intervening sequences (pMV158 family [Fig. 7]). The essential regions of the pT181-*dso* (IR-II and IR-III) involved in the interaction with the plasmid RepC initiator protein have been defined in a systematic study (312). IR-II (*nic* region) contains the RepC nick site, and the IR-III (*bind* region) is contiguous to it (Fig. 7 and 8). Whereas IR-II is conserved among plasmids of the pT181 family, IR-III is not, suggesting that the origin specificity is provided by IR-III. The proximal half of IR-III is more important for RepC recognition of the *dso* than is the distal half. In addition, the spacing and phasing between IR-II and IR-III are important for *dso* function (312). A similar picture is found in plasmids of the pC194 family. Conservation of the *nic* locus and divergence in the *bind* region can also be observed in the plasmids of the pMV158 family. In these plasmids, the distance between the conserved nick site and the direct repeats (the nonconserved *bind* locus) ranges between 14 and 95 nucleotides. The RepB protein of pMV158 binds in vitro to a dsDNA fragment containing the direct repeats (60). However, unlike the plasmids with iteron-containing origins described above,

the pMV158 direct repeats do not constitute an incompatibility determinant toward pMV158 (70). These direct repeats seem to be essential for plasmid replication *in vivo* but not for *in vitro* relaxation of supercoiled DNA mediated by the plasmid Rep protein (201).

The *nic* region contains inverted repeats able to generate one or two hairpin structures (65, 216, 254). Three different types of *nic* regions, belonging to (i) the pT181 family, similar to that of M13, (ii) the pC194 family, exhibiting similarities to ϕ X174, and (iii) the pMV158 family, with no relevant homologies to *nic* regions from known ssDNA coliphages, have been reported (Table 1) (69, 108, 228). The development of a system in which DNA strand discontinuities can be mapped with nucleotide resolution *in vivo* has allowed the identification of the nick sites of other plasmids of the pMV158 family, namely, pE194, and pFX2 (106, 328). The DNA sequences recognized by the Rep proteins to introduce the nick would be located on unpaired regions within these hairpins (IR-II in pT181; hairpin I in pMV158 [Fig. 7]), which accounts for the absolute requirement of supercoiled DNA as the substrate for replication (64, 201, 216). Genetic analysis pointed to the existence of a stem-loop structure within the *nic* region of pC194 (196). However, for the related plasmid pUB110, no transient hairpins seem to be required to initiate replication (10). *In vitro* analyses have shown that IR-II of plasmid pC221 (closely related to pT181) or hairpin I of pMV158 are sufficient for the nicking-closing activity of their respective Rep proteins. This reaction is not plasmid specific, since cognate *nic* regions are also cross-rec-

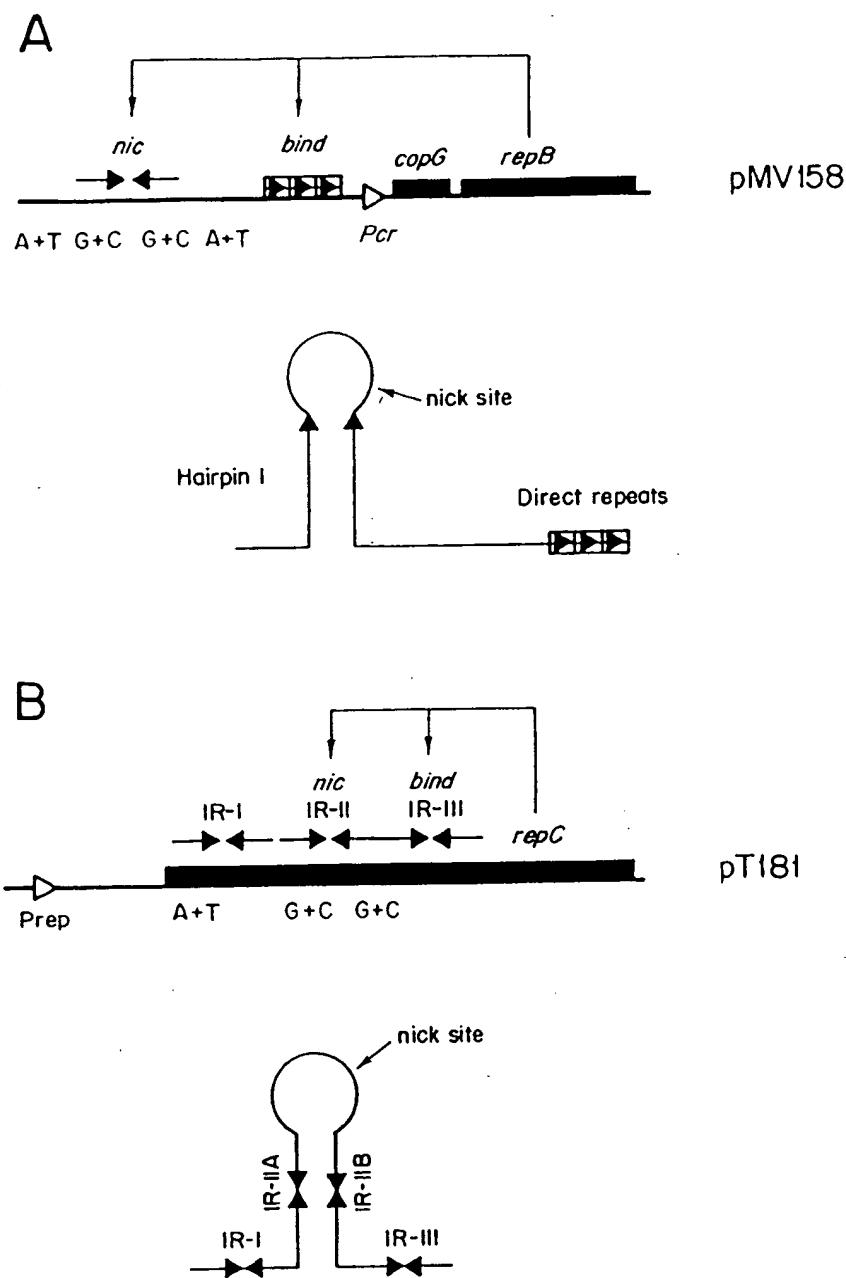


FIG. 7. Origins of replication and related regions of plasmids replicating by the RC mode, as exemplified by plasmids pMV158 (A) and pT181 (B). The *bind* and *nic* regions of the *dso* are indicated. Other symbols are as in Fig. 1.

ognized by these Rep proteins (201, 292, 332). In addition, in vitro replication of plasmid pC221 is reduced if a competing pT181-*dso* is present (332). In spite of such an in vitro recognition and extensive homologies of the Rep proteins and the *dso* of pT181 and pC221, there is no cross-reactivity between the Rep proteins and the *dso* of these plasmids *in vivo*, unless the Rep proteins are overexpressed (135).

Rep proteins. Rep proteins of the RC-replicating plasmids have DNA strand transferase enzymatic activity, so that they are able to cleave and to join plasmid DNA in a type I topoisomerase-like fashion. RepC protein of plasmid pT181 was purified and shown to have nicking activity on the plasmid *dso*

(156b). This finding, together with the presence of intracellular plasmid ssDNA and with the lack of requirement of a primer RNA for leading-strand synthesis, provided the first indications of the RC mechanism for plasmid replication (156a, 291). Rep-mediated nicking participates in the initiation of replication because the Rep proteins cleave supercoiled DNA within an unpaired sequence of the *nic* region. Cleavage and joining are essential for termination of replication (see below). Rep proteins encoded by RC-replicating plasmids have several conserved motifs which are shared with the Tra and Mob proteins (involved in plasmid transfer or conjugative mobilization) and with the Rep proteins of ssDNA coliphages and geminiviruses

TABLE 1. *nic* regions in RC replicons

Plasmid or phage	Sequence of <i>nic</i> region ^a
pT181	AAAACCGGCTACTCT/AATAGCCGGTT
pC221	AAAACCGGCTACTCT/AATAGCCGGTT
pS194	AAAACCGGtaTACTCT/AATAGCCGGTT
fd	gAgTCacgTtCTtT/AATAGtgGact
pC194	TCTTTCTTATCTTG/ATAATA
pUB110	TCTTTCTTATCTTG/ATAcat
φX174	gCTccCccAactTG/ATATTA
pMV158	GGGGGGCTACTACG/ACCCCCC
pE194	aGGGGGgTACTACG/ACCCCCC
pFX2	gttccgTACTACG/ACCCCCCa

^a Capital letters denote conserved bases. Slashes denote the sites of cleavage by the initiator protein. The positions of cleavage in pS194 and in pUB110 have not been unambiguously determined, and they are derived from homologies to plasmids of the same family.

(131, 162, 239, 310). The two more relevant motifs correspond to the enzymatic activity domain and to a putative metal-binding domain (Table 2). Curiously, this last motif is not present in plasmids of the pT181 family or in ssDNA filamentous phages. In plasmids pT181 and pC221, the phosphodiester bond 5'-ApT-3' is cleaved by Tyr-191 or Tyr-188, respectively, of the corresponding Rep proteins, which remain covalently bound to the 5'-phosphate end generated by the cleavage reaction (292). In the RepA protein of pC194, mutations in Tyr-214 abolished catalytic activity without affecting RepA-binding affinity. Furthermore, two other residues (Glu-142 and Glu-210) are also required for the catalytic activity (217). This interesting finding led to the proposal that the pC194-RepA protein contains two different catalytic residues, one (Tyr-214) involved in the covalent RepA-DNA binding for initiation of replication through transesterification and the second (Glu-210) participating in the termination step by directing the hydrolysis of a phosphodiester bond (217). The RepA Glu-210 residue was replaced by Tyr, so that termination of replication was shifted from hydrolysis to transesterification. This elegant approach led pC194-based plasmids to reinitiate after one round of replication, in a similar fashion to the replication of φX174 DNA (218). Stable covalent Rep-DNA linkage does not seem to be a general feature, since the RepB protein of plasmid pMV158 does not generate a stable covalent tyrosyl-phosphodiester bond with its DNA target (200). However, a transient bond between RepB and a DNA sequence containing the cleavage site of RepB (5'-GpA-3') could mediate the initiation of replication, resembling the situation found in the ssDNA coliphage f1 (163, 200). Analysis of the chirality of the phosphate involved in the cleavage reaction has shown that the nicking and closing reaction mediated by RepB is exerted through an even number of steps, suggesting that a covalent bond, albeit a transient one, between RepB and its target exists (202).

For plasmids of the pT181 family, studies with hybrid initiator proteins have demonstrated that a stretch of 6 amino acids, located at the C terminus of the Rep proteins, is sufficient to confer *dso* specificity, i.e., to interact with the nonconserved *bind* region of the cognate *dso* (73, 311). Although both the DNA-binding and -nicking activities of the pT181-RepC protein are required for replication, these activities are mutationally separable (73a). Protein-DNA cross-linking methods have shown that a carboxyl-terminal proteolytic fragment of the RepD from pC221 makes a specific noncovalent interac-

TABLE 2. Conserved motifs in initiator proteins of RC replicons^a

Family ^b	Sequence of:	
	Motif 2	Motif 3
φX174 cons	GRLHFHVHFM gxuHUHuxuux	VGFYVAKVYNNKSDM ugxYuakYuxkxxxx
pC194 cons	YNPHFHVLIAV yxxHUHvLUXv	ELYEMAKYSGKDSDY xxxExxKYxxKxxDU
pMV158 cons	KKAHYHVLYIA KxxHYHUUUxx	NVENMYLYLTHESKD xxxgxUxYUtxHxxxD
pT181 cons		SNRFIRIYNNKKQERK SdRFIRIYNNKqERK

^a The two motifs found in Rep proteins of plasmids representing the main plasmid families, and their comparison with those found in the initiator proteins of ssDNA coliphages (162).

^b Below each Rep protein, a derived consensus is shown.

^c Motif 2, termed the HUH motif, is thought to be the metal-binding domain of the Rep proteins. Note that this motif is not found in the Rep proteins of plasmids of the pT181 family or in the initiator gpII protein of filamentous coliphages. Motif 3 (the catalytic motif) contains the conserved Tyr residue involved in the nucleophilic attack to the plasmid DNA at initiation of replication. Capital letters denote totally conserved residues; lowercase letters denote residues conserved in many but not all proteins within the family; U and u denote hydrophobic residues; x denotes any residue.

tion with the *bind* region of its cognate replication origin (293). Such a recognition domain of this Rep protein is separated by some 80 residues from the active Tyr residue, whereas the *nic* and the *bind* sites within the *dso* are contiguous. These findings indicate that at least two separate domains of the Rep proteins could fold together in the native structure of the protein (311, 312). Resolution of the crystal structure of the Rep proteins of pC221 and/or pT181 will define the nature of the active center of these proteins. In the plasmids of the pMV158 family, the higher degree of homology found at the N-terminal region than at the C terminus of their Rep proteins suggested that their conserved N-terminal moiety would be involved in nicking activities whereas the C termini would be involved in specific *dso* recognition (69).

RepC of pT181 has been shown to function as a multimer, by demonstrating intragenic complementation between two *repC* mutant alleles. Gel filtration studies have shown that the apparent molecular weight of RepC is consistent with dimer formation (257). In RepB from pMV158, the use of glycerol gradients and analytical ultracentrifugation has shown that the protein is purified as an hexamer (60, 200), although the configuration of the protein bound to DNA has not yet been determined. RepK protein of plasmid pKYM (belonging to the pC194 plasmid family) binds the *dso* as a monomer (234).

Many of the *rep* genes have a weak Shine-Dalgarno (SD) sequence or do not have it at all. In the *repB* gene of pMV158, the existence of an atypical ribosome-binding site sequence, possibly used in *Streptococcus pneumoniae* and in *E. coli*, has been postulated (60, 72, 171). In vitro and in vivo indications that the atypical ribosome-binding site is involved in the translation of the pMV158 *repB* gene has been obtained (70). Whether those features of the translation initiation signals of the *rep* genes reflect an additional control mechanism to keep a low level of the Rep protein is not known. It is interesting that the amount of RepC protein of pT181 has been estimated to be one dimer molecule per plasmid replication event (16). Furthermore, once a RepC dimer has been used, it becomes inactive for a new round of replication because of the attachment of an oligonucleotide to one of its subunits, generating a

heterodimer, RepC/C* (255–257). Inactivation through generation of heterodimers has also been shown for pT181-related plasmids (256) and has been suggested to exist for the RepU protein of pUB110 (207). The footprints generated on supercoiled DNA by the RepC/C homodimer are different from those formed by the RepC/C* heterodimer, and whereas RepC/C was able to enhance cruciform extrusion at the *dso*, RepC/C* was not (140). Nevertheless, biochemical analysis has shown that RepC and RepC* appeared to be identical, except for the absence of the active Tyr residue in the modified protein (258).

None of the Rep proteins seems to have the HTH motif typical of many DNA-binding proteins, although a putative LZ motif exists in the RepB protein of plasmid pMV158 (60). Inspection of the DNA sequence of various *rep* genes shows two possible translation initiation signals, which would give rise to Rep and Rep' proteins (similar to the A and A* initiator proteins, respectively, of the ssDNA coliphage ϕ X174) (13). However, genetic evidence has shown that, at least for RepB of pMV158, the putative shorter RepB' protein is not sufficient to conduct replication *in vivo* (70). Plasmid pUB110 and its relative pRBH1 were reported to contain two putative SD sequences followed by a Met residue (178, 208). Overproduction of the pUB110-Rep protein showed only one major product, the one translated from the first Met residue (178). Thus, the significance of these putative Rep' proteins (if any) remains to be clarified.

Initiation and elongation of leading-strand synthesis. Little information is available on the initiation complex in RC-replicating plasmids. As stated above, the Rep proteins of pT181 and pC221 nick the *dso* and become covalently attached, by a phosphotyrosine bond, to the thymidilate residue at 3' of the nick. A strict requirement for supercoiled DNA as the substrate to initiate replication, most probably for cruciform extrusion, has been proposed (10, 64, 65, 216). In pMV158, *in vitro* RepB-dependent cleavage of the nick sequence absolutely requires ssDNA as the substrate (200, 201) and the hairpin corresponding to the *nic* region has been shown to extrude on supercoiled plasmid DNA (65, 254). For pT181, at low to moderate RepC/pT181 origin ratios, only supercoiled molecules can be used as the substrate for replication initiation (149), although specific RepC nicking activity has been demonstrated *in vitro* on linearized plasmid DNA (157). Once the Rep-DNA complex has formed, several host proteins would be required for leading-strand synthesis. To generate the replication initiation complex of pT181, the product of the *Staphylococcus aureus* chromosomal *pcrA* gene is required. PcrA has homology to helicase II and Rep helicase of *E. coli* (136). The PcrA protein is essential for cell viability and for replication of plasmid pT181. A mutation (*pcrA3*) found in the *pcrA* gene led to an increase in the proportion of nicked DNA plasmid forms, which were identified as replication initiation complexes (138). The copy number of pT181 was reduced in an *S. aureus* *pcrA3* background, and suppressor mutations were shown to map in the plasmid *repC* gene, indicating a direct interaction between RepC and PcrA (137). In addition, it is assumed that DNA Pol III-HE extends the leading strand and that due to the generation of ssDNA intermediates, SSB also participates in the replication complex.

Termination of leading-strand synthesis. Specific recognition between the Rep protein and the *dso* is required not only for initiation of RC replication but also for the termination step. Rep-*dso* recognition seems to be more stringent for initiation than for termination, because (i) abortive termination can occur at sequences similar but not identical to the origin (15, 196); (ii) part of the *dso* seems to be sufficient for termi-

nation of replication of pC194 (107); and (iii) an 18-bp sequence is long enough for termination of pT181 replication, but initiation requires a larger region which includes the RepC binding site (329). Mutational analyses have shown that the nucleotides of the right arm of the inverted repeat IR-II must be conserved for termination to occur whereas changes are allowed in some of the nucleotides of the left arm of the IR-II (330). Genetic approaches suggest that a DNA sequence, probably located 3' of the *nic* region of pUB110, is required for efficient termination of replication (10). Thus, two partially overlapping sequences, one required for initiation and the other required for termination, should be present in pUB110. For this plasmid, a single amino acid change in its Rep protein appears to reduce the ability of the Rep protein to terminate replication specifically (22).

The mechanistic information on termination of RC replication has arisen from the elegant set of experiments performed in Novick's laboratory with pT181 (141, 255, 257, 311, 312). This information has been extended for other plasmids of the pT181 family (256), as well as for pUB110 (207). The current model for RepC-catalyzed initiation and termination of pT181 replication (schematized in Fig. 8; see also Fig. 6 and Table 2) postulates that Tyr-191 of one of the subunits of a RepC homodimer exerts a nucleophilic attack at the *dso*, generating a RepC:RepC-DNA covalent complex (141, 255). Replication of the leading strand proceeds more than one full round and beyond the nick site (Fig. 8, stage 1). This would permit the IR-II hairpin at the *dso* to be exposed in both the displaced strand and the newly synthesized strand. Then the RepC subunit which was not used at the initiation step would cleave the regenerated *dso*, being covalently bound to the 5' end of the newly synthesized DNA strand (stage 2). The 3'-OH end released in this reaction (belonging to the parental strand) would exert a nucleophilic attack on the tyrosyl-phosphodiester bond generated at the initiation step (stage 3). This last reaction will release the ssDNA intermediate. The free OH group of the Tyr-191 residue of the RepC subunit involved in the initiation step attacks the nick site generated on the newly synthesized strand by the further extension of DNA synthesis (stage 4). Finally, the 3'-OH end generated would attack the tyrosyl-phosphodiester bond generated between RepC and DNA in stage 2. The end products of this reaction would be a dsDNA plasmid molecule (containing the newly synthesized plus strand) and a RepC/C* heterodimer composed of one intact RepC molecule and one modified subunit which has a short oligonucleotide covalently bound. This oligonucleotide is 12-mer on average, and it contains the 3' half of the IR-II (255). Conversion of RepC/C homodimer to RepC/C* heterodimer has been shown to be replication dependent (257). The RepC/C* heterodimer is not able to relax supercoiled pT181 DNA or to reinitiate replication, indicating that inactivation of one subunit (RepC*) may inactivate the whole molecule and that a RepC dimer is used only once in a replication cycle (255, 257). A RepU/U* heterodimer has also been found in pUB110, which would point to a general inactivation mechanism for plasmids replicating by the RC mode (208). Lack of recycling of the Rep initiator is a critical feature for a plasmid, since it would prevent overreplication (255). In pMV158, the pT181 model for termination of replication does not seem to be applicable, since RepB does not remain covalently bound to DNA (200). However, no alternative model for leading-strand termination has been proposed, and inactivation of the RepB protein has not been analyzed. A model, similar to the one proposed by Novick for pT181 RC replication, has been recently proposed for termination of ssDNA conjugal transfer

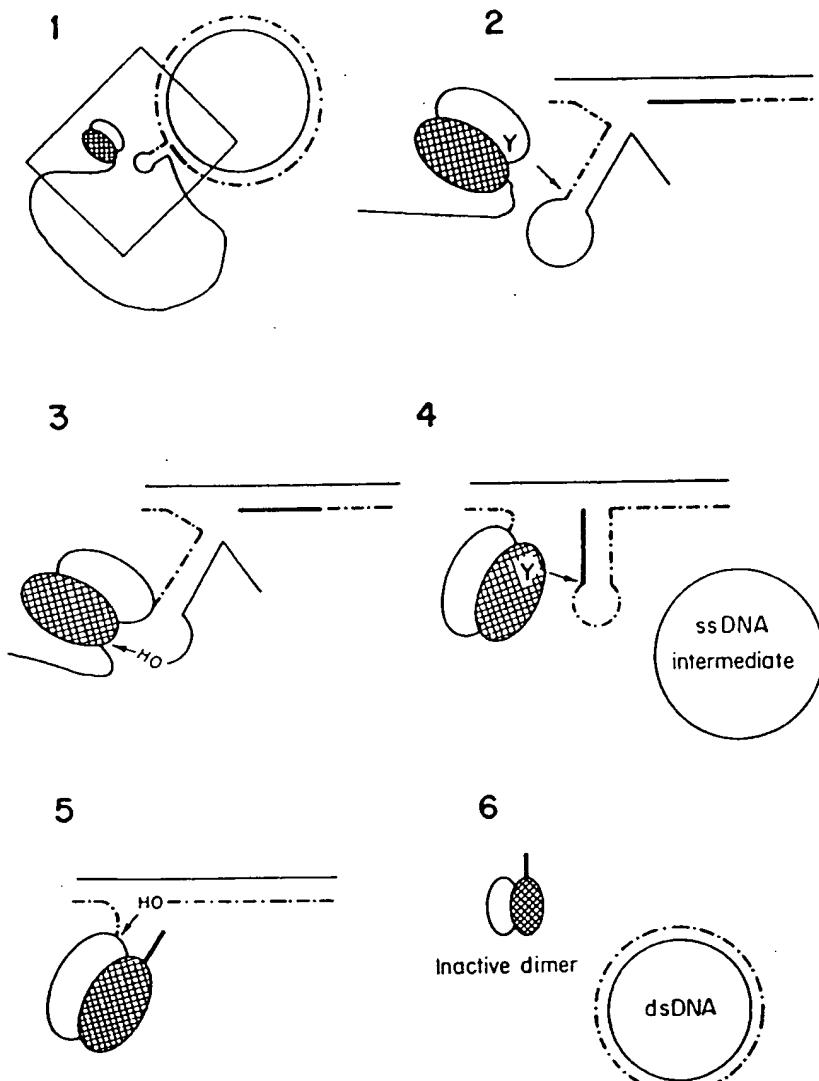


FIG. 8. Model for termination of RC replication based on results from Novick's laboratory (140, 141, 255-257). Nucleophilic attacks exerted by the OH groups of the Tyr residue of Rep (Y) or by 3'-OH groups of the DNA (OH) are indicated by arrows. Solid lines, parental DNA; broken lines, newly synthesized DNA. The thick solid line indicates the nucleotides that are newly synthesized past the reconstituted *sso* and that will remain covalently bound to the Rep protein to generate a Rep/Rep* inactive dimer. The two subunits of the RepC dimer are differently depicted.

mediated by a dimer of the Tral protein of the IncP α plasmid RP4 (240).

Replication of the lagging strand. The last stage of RC replication is the conversion of the ssDNA intermediate into a duplex plasmid DNA molecule. Lagging-strand synthesis initiates and terminates at the *sso*, which is a specific noncoding region with the potential to generate imperfect stem-loop structures. These secondary structures function in an orientation-dependent manner (109). This suggests that the ssDNA \rightarrow dsDNA conversion requires unpaired sequences within the secondary structures that constitute the *sso* (65, 68, 109). Based on sequence homologies, various types of *sso* have been reported (228). The best characterized are *ssoA* (pT181 and pC194), *ssoU* (pUB110), *ssoT* (pTA1060 and pBAA1) (75, 193), and *ssoW* (pWV01 and closely related replicons) (268). The streptococcal plasmid pMV158 has the interesting feature of bearing both *ssoA*

and *ssoU* (68, 171, 250, 306). Deletion of plasmid *sso* regions leads to reduction in the plasmid copy number (measured as dsDNA), accumulation of ssDNA molecules, and rapid loss of the plasmid in the absence of selective pressure (65, 109). Such an instability does not depend on the average number of copies of double-stranded plasmid DNA, since it cannot be overcome by mutations increasing the plasmid copy number of these deleted derivatives (68). Analysis of the DNA sequence and structure of the *ssoA* of various plasmids (228) showed the presence of two conserved unpaired regions. One of them includes part of recombination site B (RS_B), which is highly conserved in almost all of the reported *ssoA* sequences. In the center of the palindromic sequence of the *ssoA* regions, there is another unpaired region, which is conserved only among the members of each one of two different groups of *ssoA* sequences, represented by pT181-*ssoA* and pMV158-*ssoA* (65, 228). In the *ssoA* of the plasmid

group represented by pMV158, the central conserved sequence, termed CS-6, is 5'-TAGCGt/a-3' (65). The CS-6 has been shown to function as a transcriptional terminator for the synthesis of an RNA primer (see below). Mutations affecting the CS-6 sequence of the pMV158-*ssoA* moderately increase the intracellular amount of plasmid ssDNA, although the effect is not as dramatic as that of the deletion of the entire *ssoA* (165, 167).

The mechanisms of ssDNA→dsDNA plasmid conversion are poorly understood, and no known plasmid-encoded functions seem to be involved. In analogy to ssDNA coliphages, a pRNA is assumed to be needed to initiate lagging-strand synthesis. Experimental evidence suggests that in most cases, ssDNA→dsDNA conversion is mediated by RNAP, since ssDNA accumulates *in vivo* in the presence of rifampin (27, 74, 165). *In vitro* studies have shown that an ssDNA fragment containing the pMV158-*ssoA* was able to promote RNAP-directed synthesis of a 20-nucleotide pRNA. This ssDNA fragment specifically bound RNAP, with this binding requiring an intact RS_B sequence (166).

The *ssoW* of plasmid pWV01 is made up of two inverted repeats (IR1 and IR2). Conversion of ssDNA→dsDNA from an intact *ssoW* seems to require the host RNAP (173). However, deletion of the IR2 resulted in a reduced and RNAP-independent conversion. These findings, together with the homologies between the *ssoW*-IR1 and the lagging-strand origin of φX174, led to the proposal of an alternative mechanism of pWV01 lagging-strand priming mediated by the host primosome (173, 268).

In addition to the host RNAP, DNA Pol I is involved in lagging-strand replication both in initiation and in termination (76, 166). Detection of ssDNA intermediates may be masked because the amount of ssDNA accumulated depends upon the specific host-plasmid pair and, in their natural hosts, single-stranded→double-stranded plasmid DNA conversion is so efficient that the half-lives of ssDNA intermediates are too short to be detectable (68, 108, 306). Nevertheless, implementation of an *in vitro* system for replication of ssDNA (23, 210) facilitated the determination of the RNA-DNA transition points, which corresponded to regions including the above-mentioned conserved central sequences (CS-6) of the secondary structure of the *sso* regions (74). The use of pMV158-based vectors with no *sso* indicated the existence of an alternative pathway of conversion in which plasmid-encoded antisense RNAs, annealed to the complementary region on the ssDNA intermediates, may act as primers in a process mediated by the host RecA protein (194).

Synopsis. Replication by the RC mechanism is widespread among multicopy plasmids from the *Archaea* and *Bacteria*. The 3'-OH end required for initiation of replication is provided by the site-specific nicking activity of the plasmid-encoded Rep protein on one of the parental plasmid strands. The DNA substrate for the Rep-mediated nicking has to be in a single-stranded configuration. This can be achieved by Rep-facilitated extrusion of a cruciform structure encompassing the *nic* region of the origin, where the nick site is unpaired. Leading-strand synthesis is terminated by various specific strand transfer reactions, also mediated by the Rep protein. After completion of leading-strand synthesis, the Rep protein is inactivated and plasmid ssDNA intermediates are generated. RNAP-directed synthesis of a short RNA primer initiates lagging-strand replication. In this step, only host-encoded proteins are involved.

CONTROL OF PLASMID REPLICATION

Although plasmid copy number may vary in different bacteria, within a given host, and under fixed growth conditions, any particular plasmid has a characteristic copy number. This is achieved by plasmid-encoded control elements that regulate the initiation of the replication step. Control systems maintain the rate of replication in the steady state at an average of one replicative event per plasmid copy and cell cycle by correcting deviations from the average copy number in individual cells. To define and to maintain the copy number, plasmids use negative regulatory circuits (251). The genetic analysis of replication control mechanisms was first performed for plasmid R1 through the isolation of mutants which exhibited an increased copy number (225). Characterization of these mutants indicated that the determinants of copy number control resided in the plasmid itself and that negative regulators (inhibitors), acting at the initiation step, were involved in this control. A model of control of replication modulated by negative effectors was first proposed and described in quantitative terms by Pritchard et al. (252). When a plasmid colonizes a new host, the concentration of these negative regulators should be negligible. This seems desirable for successful establishment, since unhindered plasmid replication would permit the normal copy number to be reached in a short time. However, once the characteristic copy number is reached, keeping the average copy number in the population requires adjustments to fluctuations in this value in individual cells. The control systems do just that by either increasing or decreasing the rate of replication per plasmid copy and cell cycle. Individual plasmid copies are selected for replication at random from a pool that includes replicated and nonreplicated copies. However, mechanisms that counterselect newly replicated molecules exist, e.g., hemimethylation and supercoiling (3, 224).

Control of replication by negative regulators and random selection of plasmids for replication have an additional consequence: two plasmids with identical replicons are unable to stably coexist within a given cell in the absence of selective pressure. This leads to segregation of plasmids within the host population, a phenomenon known as plasmid incompatibility. The inhibition of plasmid replication, associated with an increase in the gene dosage of copy number control genes, has been used to identify these genes (reviewed in references 12, 51, 155, 222, 227, and 251).

Control of replication by inhibitors would require that they could "measure" the concentration of plasmid copies within the cell. This could be achieved by an unstable inhibitor expressed constitutively or by a stable inhibitor synthesized shortly after each initiation event (252). Any of these alternatives would reduce the initiation frequency after each initiation event and would increase or decrease the rate of initiation of replication when the average copy number is, respectively, lower or higher than required. When the frequency of initiation is determined by the level of an initiator protein, one mechanism for controlled plasmid replication (unlike phage replication) would be to inactivate the initiator protein after each replication event (255, 319).

Mechanisms controlling replication have been studied in various systems (reviewed in reference 293a), and several types of inhibitors have been detected: (i) antisense RNA (ColE1, R1, and pT181); (ii) both an antisense RNA and a protein (pMV158 and pIP501), and (iii) DNA sites for binding initiator proteins (F, P1, RK2, and R6K). A schematic representation of these regulatory loops is depicted in Fig. 9. Control of replication by a protein (lambda-dv) is well documented and quantified (222), but it has not been described in natural plasmids

and is not discussed here. Accessory elements that can also modulate replication but that are not directly involved in controlling the initiation frequency are not discussed either.

Control by Antisense RNA

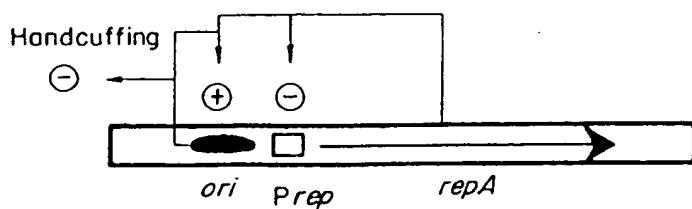
Interactions between RNAs can modulate the availability of either a pRNA or the level of a rate-limiting Rep protein, which are essential for replication. Antisense RNAs controlling plasmid copy number are complementary to a region in the 5' end of the target transcript (preprimer RNA for replication or *rep* mRNA) and are termed "countertranscribed RNAs" (ctRNAs) (227, 228, 309). In some cases, inhibition by ctRNAs is achieved in the region of pairing with the target RNA. In other cases, formation of the inhibitor-target duplex leads to a conformational change in the target transcript, which is rendered inactive. Examples of these mechanisms of control are described below.

Control of primer RNA processing: plasmid ColE1. ColE1 was the first example where control of replication was found to be exerted by RNA (170, 296). In fact, the analyses of copy number control circuits of ColE1 led to the identification of antisense RNAs, a milestone discovery in molecular biology. *trans*-acting antisense RNAs permit the silencing of specific genes by pairing to mRNA molecules. The bases of the control of ColE1 replication are well known (reviewed in references 47 and 82). Initiation of replication is primed by a 550-nucleotide transcript, RNA II, which is made by RNAP and is specifically processed by RNase H. This processing is required for the transition of RNA to DNA synthesis, since it generates a 3'-OH end on which Pol I initiates the synthesis of the leading strand. The availability of this 3'-OH end is rate limiting for initiation and is modulated by an RNA of 108 nucleotides, the antisense RNA I, which is entirely complementary to a region in the 5' end of the pRNA II. Hybrid formation between the antisense and the preprimer RNA alters the overall secondary structure of the preprimer. As a consequence of this conformational change, the 3' end of the preprimer cannot hybridize to the DNA. This, in turn, inhibits the initiation of replication because, in the absence of DNA-RNA hybrid formation, the preprimer is not processed by RNase H. Genetic and biochemical analyses showed that interactions of the antisense RNA with the complementary sequences of the preprimer occur initially at regions exposed as single-stranded loops on both folded RNA molecules. Formation of this initial contact (or "kissing" complex) is the rate-limiting step of the interaction and leads to the full annealing of the antisense RNA I with the preprimer RNA II, in a process that starts at the 5' end of the RNA I. The RNA I is synthesized from a constitutive promoter (therefore its level is proportional to the plasmid copy number) and is unstable. An auxiliary role is provided by a small basic protein, termed Rop or Rom (276, 304). Rop interacts with the antisense RNA and with the preprimer, increasing the efficiency of formation of the "kissing" complex and thus decreasing the efficiency of replication. The gene encoding this protein is not an incompatibility determinant, but it can reinforce the antisense RNA I-mediated incompatibility.

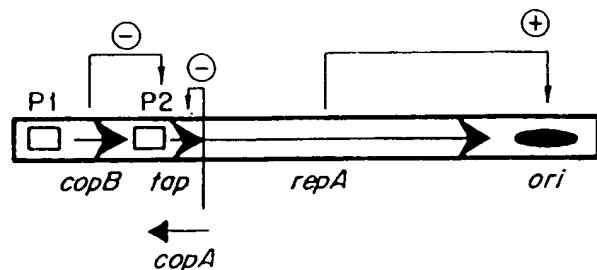
Copy number control of plasmid R1. Copy number control in R1 is exerted at the level of RepA synthesis, which is modulated by the products of the copy number control genes, *copB* and *copA* (reviewed in references 224 and 309). The products of *copB* and of *copA* inhibit *repA* expression at the transcriptional and posttranscriptional levels, respectively. RepA is rate limiting for replication, acts preferentially in *cis*, and cannot be reused. The main copy number control gene is *copA*, while *copB* plays an accessory role. *copA* is transcribed from a con-

stitutive promoter, and its product is an unstable (half-life, 2 min) RNA, CopA, which is complementary to a leader region (termed CopT) of the *repA* mRNA. Hybrid formation between CopA and its CopT target is also achieved through formation of a "kissing" complex. The CopA-CopT hybrid inhibits RepA synthesis by hindering the translation of a leader peptide-encoding gene, *tap*, which is translationally coupled to *repA* (26). The efficiency of inhibition depends on the affinity of kissing-complex formation (reviewed in reference 226). Mutations in *copA* that decrease the efficiency of the complex formation increase the copy number, but replication still is controlled. However, complete inactivation of CopA leads to uncontrolled (runaway) replication (reviewed in references 224 and 226). The duplex CopA-CopT is specifically cleaved by RNase III, and this cleavage seems to be an important step in the control of RepA synthesis (25). Mutational analysis performed at *copA* showed that the structure of the major stem-loop in CopA is important for its function. The presence of bulges in the stem region of CopA is required for rapid duplex formation with its CopT target in vitro, as well as for effective inhibition in vivo (126). In addition, the presence of bulged nucleotides in the CopA stem seems to result in a structural destabilization that plays a protective role against degradation by RNase III (125). Structural and functional studies on CopA have permitted us to draw a direct correlation between gene dosage (plasmid copy number) and CopA levels. This determines a constant rate of replication, independent of the copy number of the plasmid or the growth rate of the host. Two consequences derive from these results: (i) the frequency of plasmid replication per copy is >1 when the number of plasmid copies per cell is below the average and <1 when the number of copies is above the average; and (ii) the copy number of R1 increases with decreasing host growth rate. A constant rate of replication per cell, independent of copy number, will slowly correct deviations from the average frequency of plasmid replication (one per plasmid copy per cell cycle), and therefore it will actively maintain the average copy number constant (222). The second regulatory element in R1, the CopB protein, plays an auxiliary role under normal circumstances. This small dimeric and basic protein represses transcription of *repA* from a promoter, P2, which is located downstream of *copB* (Fig. 9). Under steady-state conditions, CopB is present at saturating concentrations, fully repressing transcription from P2 to a basal level. Under these conditions, the *repA* mRNA is synthesized mainly as a polycistronic *copB-tap-repA* RNA. However, under conditions in which the copy number drops or is low because of the early stages of plasmid establishment, the CopB-mediated repression is not efficient. In these circumstances, the P2 promoter is derepressed and *repA* is also transcribed as a *tap-repA* mRNA. This leads to a transient increase in the transcription rate of *repA* and, as a consequence, to a temporal increase in the frequency of plasmid replication. Thus, derepression of the P2 promoter would play an important role during the early phase of plasmid establishment or in situations in which the copy number falls below the wild-type level (224, 320). However, a computer-based simulation suggested that the auxiliary control loop mediated by CopB should have only a marginal effect not only under steady-state conditions but also during plasmid establishment (259).

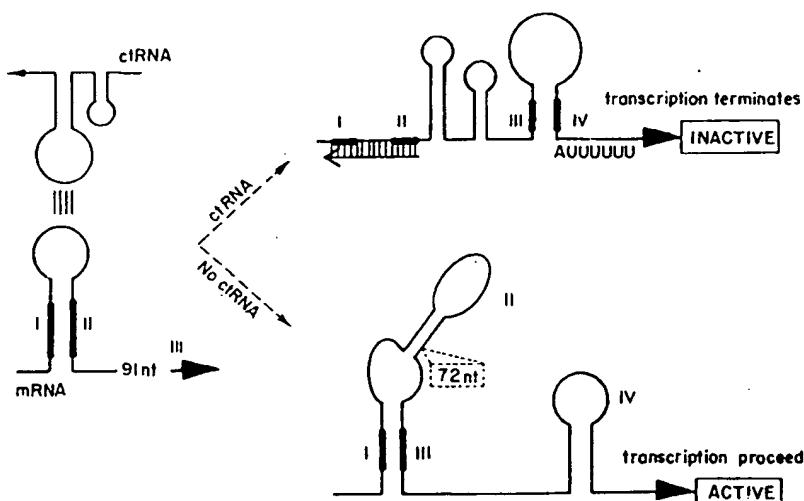
Other instances of control by antisense RNAs. There are other examples in which replication is controlled by antisense RNA. For instance, in plasmids of the IncI α and IncI β groups, control of replication is modulated by a short ctRNA that inhibits, as in R1, synthesis of a rate-limiting Rep protein, which, in turn, is translationally coupled to the synthesis of a



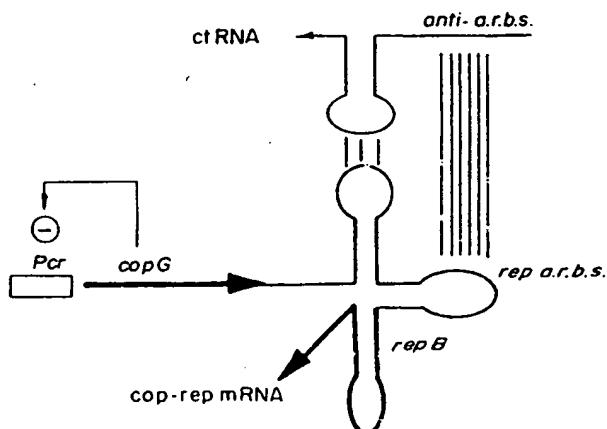
P1



R1



pT181



pMV158

FIG. 9. Control of plasmid DNA replication. Examples of control by Rep DNA-binding sites (plasmid P1), by antisense RNAs (plasmids R1 and pT181), and by a dual system (plasmid pMV158) are depicted. Transcripts are shown as continuous lines, with arrowheads indicating the direction of synthesis. mRNAs are shown with thicker lines than antisense RNAs; countertranscribed RNAs (thin lines with small arrowheads) are indicated. Other symbols as follows: solid ellipses, origins; rectangles, promoters; a.r.b.s., atypical ribosome-binding site; parallel vertical lines, mRNA-ctRNA interactions; plus, positive interactions; minus, and inhibitory RNA-RNA or protein-DNA interactions.

leader peptide. Inhibition by ctRNA is exerted at the posttranscriptional level by hindering the synthesis of the leader peptide. In addition, ctRNA prevents the formation of an activator RNA pseudoknot that is required for efficient synthesis of the Rep protein (reviewed in reference 309).

Antisense RNA has been involved in the silencing of the *ori-γ* of plasmid R6K (243, 244). Replication from this origin requires the synthesis of an activator RNA that starts within the seven iterons of the origin and is silenced by the antisense RNA. The replication-initiator protein, π , seems to favor interactions between these transcripts. This regulatory system is independent of the one modulated by iterons. R6K is not the only theta-replicating iteron-containing plasmid in which antisense RNA plays a role in regulating replication. In plasmid ColE2, a ctRNA complementary to the leader *rep* mRNA has been involved in the control of replication at the posttranscriptional level through generation of a ctRNA-mRNA hybrid that sequesters a sequence(s) essential for Rep synthesis other than the SD region (287, 323).

Direct inhibition of Rep synthesis: blocking *rep* translation. A direct way to control plasmid copy number by a ctRNA is the inhibition of Rep synthesis by blocking the accessibility of the ribosomes to the *rep* mRNA translation initiation sequences. This type of control has been proposed for some plasmids replicating by the mechanisms of strand displacement (R1162) (154) and RC replication (pMV158) (67). In both instances, the main *inc* determinant is located in a region encoding a ctRNA, and the putative signals for initiation of *rep* translation are placed on a predicted loop of the *rep* mRNA. In the case of R1162/RSF1010, the frequency of initiation is determined by the level of the RepC DNA-binding protein (112, 153). Expression of *repC* is regulated at the transcriptional level by the first gene of the *repC* operon (179) and at the translational level by a ctRNA that is complementary to the translation initiation signals of *repC* (154). Which of these two regulatory mechanisms makes the RepC synthesis rate limiting is not yet well understood. In the RC-replicating plasmid pMV158, a ctRNA has been proposed to inhibit the translation of the *repB* gene by direct interaction with the initiation of translation sequences present in the *repB* mRNA (67, 71, 72). Secondary structures in the ctRNA, other than that corresponding to the transcriptional terminator, have not been found. Plasmids lacking the DNA region encoding this terminator and the complementary structure on the *cop-rep* mRNA are still sensitive to the wild-type ctRNA supplied in *trans*, suggesting that formation of a kissing complex may be important but is not essential for the generation of the hybrid ctRNA-mRNA. A more complex regulatory loop in pMV158, which involves the joint participation of the ctRNA and of a repressor Cop protein, was described later (see below). Analogous genetic structures in the control-of-replication region have been found in plasmids of the pMV158 family (69). A two-component system (Cop protein and antisense RNA) controlling the replication of plasmid pIP501 has also been proposed (35; see below). For plasmid pUB110, synthesis of the initiator RepU protein seems to be controlled by two antisense ctRNAs that interfere with *repU* translation, since the putative ctRNAs are complementary to *repU* initiation of translation sequences (178), although a mechanism of control similar to that of pT181 has also been suggested (11, 249). However, since the synthesis of RepU is autoregulated (207), a more complex control level may have to be considered.

Transcriptional attenuation: the pT181 paradigm. A singular copy number control mechanism, involving ctRNAs, has been well established for pT181 (119, 169, 227, 228). The current model for the control of replication of pT181 (refer-

ences 227 and 228 and references therein) proposes that synthesis of the Rep initiator is limited by two small ctRNAs (having the same 5' end but different 3' ends), which are complementary to the untranslated 5' end of the *rep* mRNA (44, 119, 227, 229). The initial kissing-complex formation between ctRNAs and *rep* mRNA would take place through nucleotides exposed in complementary loops of hairpin structures that can be formed on both RNA species. Formation of the ctRNA-mRNA hybrid would lead to a conformational change in a region of the mRNA far apart from the region of complementarity. The new mRNA configuration would be such that a new hairpin ending in an A(U)₆ sequence could form. This secondary structure resembles a Rho-independent transcription terminator and therefore can truncate the *rep* mRNA (230). Thus, transcriptional attenuation (as found in the control of many biosynthetic operons) (161), rather than blocking translation initiation, is the mechanism for controlling the copy number of pT181. A similar control, through premature termination of the *rep* mRNA promoted by the indirect action of a ctRNA, has been proposed for plasmids of the pIP501/pAMβ1 family (33). For pIP501, it has been shown that steps occurring before stable ctRNA-mRNA duplex formation are sufficient to attenuate mRNA synthesis (34).

Control by both Transcriptional Repressor and Antisense RNA

Control by the joint action of a transcriptional repressor and an antisense RNA has been characterized for plasmids R1, pMV158, and, more recently, pIP501, although differences in the role of the components have been found (35, 69, 71, 72, 224). As described above, the main control in R1 is exerted by the ctRNA CopA whereas CopB plays an auxiliary role. In fact, deletion of *copB* leads only to a moderate increase in plasmid copy number, and the *copB* gene cloned into a compatible replicon does not exhibit incompatibility toward R1.

In the case of pMV158, a transcriptional repressor, CopG, binds to and represses transcription from a single promoter for both the *copG* and *repB* genes (66). Mutations or deletions in *copG* lead to an increase in the plasmid copy number (7), and *copG* exerts only a weak incompatibility toward plasmids with the pMV158 replicon when cloned, under its own transcription and translation signals, into a high-copy-number compatible vector (67). A second element involved in copy number control is a small countertranscribed RNA, RNA II, which is complementary to a region of the *cop-rep* mRNA between genes *copG* and *repB*. *mall* is a strong *inc* determinant when supplied in *trans* at high gene dosage. Mutations that abolish the synthesis of the ctRNA also lead to an increase in copy number, but replication is still controlled by CopG. However, in this case, great fluctuations in copy number within individual cells were observed (71). No significant incompatibility toward plasmids with the pMV158 replicon was found when the *mall* gene was cloned in a low-copy-number plasmid (71). The control circuit elements (*copG* and/or *mall*) have been cloned at a physiological gene dosage (i.e., at a copy number similar to that of the wild-type plasmid). In this case, a strong incompatibility toward pMV158 was found when the entire circuit was provided in *trans* whereas either component cloned separately exhibited weak (*ctnall*) or no (*copG*) incompatibility. The hypothesis derived from these experiments was that the control of replication of pMV158 is exerted by the entire regulatory circuit rather than by a hierarchy of any of the components (71). Due to the genetic organization of the plasmids of the pMV158 family, a similar regulatory circuit seems to exist in all of them (61).

The theta-replicating plasmid pIP501 seems to share features with R1 and pMV158 in their replication control circuit. Similarly to R1, the CopR protein of pIP501 is not a regulator of its own synthesis but represses transcription from the essential downstream *rep* promoter (31). However, and unlike R1, the CopR-inhibited *rep* promoter is not fully repressed, a situation resembling pMV158. The second element regulating pIP501 copy number is the unusually long-lived antisense RNA III (35). The stability of this antisense RNA III poses the problem of corrections of down-fluctuations in copy number, since a decrease in the number of plasmid copies will not be rapidly followed by a concomitant reduction in the levels of the inhibitor. This would lead to plasmid loss, which has not been observed (139a). To solve this apparent paradox, a model similar to the one proposed for pMV158 (71), in which a reduction in the pIP501 copy number would lead to derepression of the *rep* promoter due to reduction in the CopR levels, has been postulated (35).

The recent finding that the RepU protein of plasmid pUB110 regulates its own synthesis, thus introducing an additional control for plasmid copy number (207), would represent another example of dual control (transcriptional and translational regulation). Mechanistically, the situations for pUB110 and the pMV158 may be indistinguishable, which may point to a more general control of replication mechanism in these plasmids replicating by the RC mode.

Control by Iterons

The Rep proteins of the theta-replicating iteron-containing plasmids are either autoregulated or under transcriptional control, and the iterons play a role in the control of plasmid copy number (reviewed in references 51, 155, and 223). The role of the iterons was initially described for plasmid F, based on the observation that *oriS*-dependent replication was inhibited when the iterons of *oriS* were cloned on a compatible plasmid (295). Similar situations were also found for other iteron-containing plasmids, such as P1 or R1162/RSF1010. The extent of inhibition in P1 was found to be proportional to the number of cloned iterons (238) whereas in R1162 the inhibition was abolished by overproduction of the rate-limiting Rep protein (153). These observations led to the formulation of the titration model (303). The model assumed that the Rep protein is rate limiting for initiation and that iterons titrate the Rep protein, thus limiting the frequency of initiation. A paradox developed when the Rep proteins of most iteron-containing plasmids were found to be autoregulated, which implies that titration could be overcome by derepression (49). Furthermore, in other systems (R6K and RK2), the amount of Rep protein within the cell was apparently too large to be limiting for saturation of iteron binding (88, 190). This led to the modification of the initial model and to the proposal that different forms of the Rep protein were involved in autoregulation and in initiation, so that initiator titration does not cause derepression (302). A computer model that adjust to this hypothesis has been developed (321).

A second solution to the titration/autoregulation paradox was provided by the model that Rep molecules bound to the P1 iterons were still able to repress the *repA* promoter (49). The model was based on the ability of Rep proteins to bind to two iterons simultaneously (in both P1 and R6K) (204). This property of the Rep proteins also provided the basis for a new model of negative control of replication called the "steric hindrance" (238) or "handcuffing" (155, 191) model. According to this model, when Rep proteins bind to and saturate the iterons of the origin, initiation occurs if the plasmid copy number is

low. As the number of copies increases, Rep molecules bound to the iterons of one origin begin to interact with similar complexes generated on other origins. As a consequence, plasmid molecules pair through Rep-Rep interactions, causing a steric hindrance to the function of both origins ("handcuffing"). The plasmid pairs are apparently separated during subsequent cellular growth, and the initiation potential of individual molecules is restored. According to this model, it is the iteron concentration, rather than the level of Rep expression, that determines the rate of replication. The question of the role of autoregulation remains open, and no simple answer has been provided. As mentioned above, in RK2 and R6K, a substantial decrease in the levels of Rep protein has no effect on the rate of plasmid replication. However, in P1, a twofold reduction in the level of the Rep protein abolishes replication and a fourfold increase can lead to inhibition of replication (238). Similarly, in R6K, a twofold increase in initiator concentration is inhibitory to initiation. These data indicate that autoregulation is important, at least in some cases, to keep an optimal concentration of the initiator protein. It has recently been suggested that in particular plasmids in which initiators are limiting, initiator titration and initiator pairing (handcuffing) could be working together (51).

Some of the replicons containing iterons in the origin also contain iterons outside this region, which express incompatibility and further reduce the rate of replication (237). In principle, the probability of Rep-mediated iteron pairing greatly increases in these replicons, since it can occur in *cis* by DNA looping and in various combinations in *trans*. Since control systems should measure the copy number, and since intramolecular pairing is independent of this parameter, this pairing cannot correct deviations from the average copy number. However, intramolecular pairing can regulate the level at which the copy control operates (discussed in reference 223). A different question is whether the iterons within and outside the origin region are equally effective as replication control elements. Recent data on RK2 and P1 plasmids indicate that iterons outside of *ori* are more effective as negative regulators of replication than are iterons present in the origin region, probably due to differences in the quantitative and structural features of the two sets (5, 273). It has been proposed that in RK2 these auxiliary iterons stimulate initial pairing between origins. The data for P1 indicate that the concentration of the auxiliary iterons rather than their relative arrangements is the deterministic factor for replication control (51).

The role played by Rep proteins of iteron-containing plasmids in the control of replication and in autoregulation implies that specific *rep* mutations affecting protein-protein or protein-DNA interactions could increase the plasmid copy number. In R6K, copy-up mutations affecting the plasmid initiator protein have been obtained (87). Most of the copy-up mutations affected a 32-amino-acid region placed between the LZ motif and the putative DNA-binding domain of the π protein, a region that seems to be involved in high-order oligomerization of the initiator protein (199). Mutations that affect the LZ motif of the Rep protein and lead to copy-up phenotypes in pSC101 have been described (133). Copy-up mutants of plasmid RK2 containing mutations encoding TrfA variants defective in binding to DNA have been isolated, indicating that copy number control is modulated by TrfA-DNA interactions (46). Copy-up mutations in the *trfA* gene have also been selected as intragenic suppressors of thermosensitive *trfA* mutations. Some of these copy-up mutations were proposed to reduce a strong protein-protein inhibitory interaction induced, at the restrictive temperature, by the thermosensitive mutation (114).

Hemimethylation and Regulation of Plasmid Replication

The heptamer repeats containing the methylation sites in the origin of replication of plasmid P1 constitute the target of the host protein SeqA, involved in sequestering hemimethylated *oriC* into the bacterial membrane (37). Methylation seems to regulate replication at two different levels. First, hemimethylation would exert a negative regulation through *ori*-membrane interactions. The newly replicated, hemimethylated DNA is sequestered in the cell membrane, thus preventing reinitiation. The sequestration is relieved as the hemimethylated DNA undergoes new methylation (51). At a second level, methylation increases the initiation efficiency both *in vivo* and *in vitro*. This stimulation could be due to enhanced bending or unwinding of the DNA or to recycling of initiators (3, 51).

Synopsis

Plasmid DNA replication occurs coupled to the cell cycle of the bacterial host in such a way that a fixed concentration of plasmids is maintained in the bacterial population. This is achieved by mechanisms that monitor the initiation frequency and adjust this frequency to an average of one replication per plasmid copy and cell cycle. The control of plasmid replication is plasmid encoded and is performed by molecules (antisense RNA, proteins, or DNA sequences) that inhibit the initiation of replication in a dose-dependent way. This inhibition can limit the level of active Rep protein, the availability of the primer for the leading strand, or the number of active origins. Control of plasmid replication may be exerted (i) by a single inhibitor, (ii) by a main controller and an auxiliary element, or (iii) by the concerted action of two inhibitors (a protein and an antisense RNA or two sets of iterons). In general, any model on control of plasmid replication should explain how the average plasmid copy number is maintained in a growing population and how fluctuations on this average are corrected.

SUMMING UP: DIFFERENCES AND SIMILARITIES IN PLASMID REPLICATION MECHANISMS

In spite of the progress that has been made in the study of plasmid replication and its control, there are major gaps in our knowledge of the various replication systems. Initiation of replication demands melting of the origin and interactions between plasmid-encoded and host-encoded factors, which are in general only poorly understood. Melting of the origin can be stabilized by either manifesting the cryptic ssDNA-binding activity of the initiation proteins (DnaA and R6K- π) or by the formation of intramolecular secondary structures (e.g., cruciform extrusion). The configuration of the plasmid origins indicates that DNA curvature and Rep-enhanced origin bending seem to be a common but yet not fully explored feature (81, 156c, 205, 206, 283). DNA deformations at the origin may provide an appropriate configuration for the initiation event: cruciform extrusion and nicking by the initiator for RC-replicating plasmids versus assembly of the initiation complex and synthesis of an RNA primer for the other replicons. The role of host-encoded proteins in the generation of the initiation complex is relatively well defined for several plasmids replicating by the theta mechanism, but little is known for plasmids using the RC mode (apart from the PcrA helicase from *S. aureus* in pT181 replication) (135–138). Definition of the specificity of the interactions between Rep proteins and host replication factors is relevant to our understanding of the ability of plasmids to colonize different hosts. The convergence of structural studies with the functional analysis of initiators and in-

hibitors of DNA replication is an important area of future development. Host replication factors and plasmid initiators form part of the nucleoprotein complex that initiates plasmid replication. The structural definition of these macromolecular assemblies will provide mechanistic information relevant to our understanding of the initiation of plasmid replication and the interrelations between plasmids and their hosts. Since the continuity of the parental DNA strands is kept in plasmids replicating by the theta and strand displacement mechanisms, superhelical tensions accumulate during the elongation stage. A role for topoisomerases in the elongation of DNA synthesis and in the separation of both catenated daughter molecules has been documented (8, 9, 117, 245, 246, 327). Such a role may not be required for plasmids replicating by the RC mechanism, although the relaxed DNA molecules (which are the products of RC replication) must be supercoiled by DNA gyrase before they become a substrate for a new round of replication. Finally, information is scarce on three important plasmid replication events: (i) the role of transcription through origins to create waves of supercoiling, (ii) the structural changes introduced by chaperones that result in activation of Rep proteins, and (iii) the mechanisms of possible Rep inactivation, except for several RC-replicating plasmids (255, 256).

The study of specific signals involved in the termination of replication of theta-type plasmid replicons is a matter of growing interest. Elucidation of the structure of termination proteins (RTP and Tus) acting at specific termination sites has opened new avenues to perform detailed mechanistic analysis of this stage of replication (19).

Concerning lagging-strand synthesis, only host factors are thought to be required in plasmids replicating by the RC and theta modes. This contrasts with the situation for plasmids replicating by the strand displacement mechanism, in which the same plasmid-encoded primase and helicase proteins are involved in replication of both strands, in a continuous process that starts in two symmetrical origins and proceeds in opposite directions (263, 266). In this latter plasmid category, uncoupling synthesis of both DNA strands may occur, leading to the generation of ssDNA plasmid intermediates. However, unlike plasmids replicating by the RC mechanism, there is no strand specificity in the ssDNA intermediates generated during the strand displacement replication.

Control of plasmid replication is one of the key features of these extrachromosomal elements. Such control is always exerted at the initiation stage, perhaps because the initiation events, as opposed to the elongation and termination steps, are invariably replicon specific. The role of RNA inhibitors in the control of replication is relatively well understood (226, 309). However, replication control modulated by iterons is less well known and is an object of intense research (51, 155). Many of the initiators that bind to iterons autoregulate their own synthesis. How this autoregulation influences the frequency of initiation is important to a full understanding of control of replication in these plasmids. Interestingly, the strategies used to control plasmid replication do not correlate with the mechanism by which the plasmid replication initiates: replication initiation by different mechanisms may have similarities in control circuits (pIP501 and pMV158; pIP501 and pT181; and R1162 and pMV158). Finally, although the elements controlling replication have been identified in many instances and their modes of action have been described, there has not been much efforts to understand the control in terms of kinetics, except for a few cases (12, 223).

CONCLUDING REMARKS AND PROSPECTS

The main emphasis of this review has been to show that plasmids are important models with which the combination of genetic, biochemical, and molecular approaches have revealed many biological phenomena. An important focus for molecular biologists studying plasmids has been on the mechanisms involved in their replication and the control of this process. Replication studies have revealed a variety of mechanisms that are used to replicate circular or linear plasmids. Plasmids contribute with specialized sequences and functions to their own replication but, in general, use extensively the replication machinery of the host. Replication of these genetic elements therefore provides a window to gain information on the communication between plasmid- and host-specific replication machinery. Buried in these interrelationships is the ability of plasmids to colonize different hosts (i.e., their host range), which has important ecological and biotechnological implications.

Plasmid initiator proteins have drawn much interest, and the studies on these activators have been focused on the specific protein-protein and protein-DNA interactions involved in the formation of an active initiation complex. Although genetic and biochemical analyses have allowed the isolation and characterization of Rep variants affected in their interaction with DNA, the relevant protein domains involved in contacting DNA are not clearly known in most cases. Concerning protein-protein interaction, the available information indicates that the initiation complex is formed by multiple copies of the initiator protein interacting not only with the DNA in the origin region but also with each other and with host initiation factors, not to mention the role of membranes in DNA replication. The analysis of the Rep protein of plasmid pPS10 has given important clues on protein motifs involved in these interactions, although the picture is far from complete. Atomic resolution of the structure of an initiator and of a multiprotein-DNA initiation complex will be an important step toward a clear understanding of these interactions and the changes introduced in the target DNA. This could be done not only through the resolution of crystal structures of protein-DNA complexes but also through the use of many other available physicochemical approaches (such as nuclear magnetic resonance, light and neutron scattering, circular dichroism, and analytical ultracentrifugation, to name a few). Some steps in these directions have recently been taken for the RepA protein of plasmid P1 (50).

Studies on the control of plasmid replication have revealed the role played by small antisense RNAs in the processing of RNAs or in the specific inhibition of Rep-protein synthesis at the transcriptional or translational level. The biotechnological and clinical implication of this important discovery is well known and reveals, once more, the important practical implication of basic studies. The different ways in which antisense RNAs modulate the control of replication are well established. A less well known mechanism is the control of replication modulated by iterons, and this is an active field of research.

In contrast to molecular biologists, microbial ecologists have focused their attention mostly on lateral spread and persistence of plasmids in natural environments. However, in this area there is a lack of application of molecular biology techniques, which may result in a biased identification and characterization of plasmids isolated from new environments or from environments which have not been subjected to extreme selective pressures. Molecular analysis of plasmid replication and its control occurring in bacterial populations growing under natural conditions should be considered. Growth phases other than the exponential phase should also be considered and analyzed, and new methods to study plasmid replication in

mixed bacterial populations and in bacteria growing on microfilms should be developed. Analysis of the so-called cryptic functions harbored by plasmids may reveal new areas of potential interest. A study on the presence of plasmids in the nonculturable bacteria, the vast majority of the microorganisms contributing to the biodiversity, remains to be done. The interests of biotechnologists in plasmids harboring "food-grade" selectable markers, in the mechanisms of stable inheritance, and in microbially aided improved crops are very different from those of people working in hospitals, who are more concerned with clinical outbreaks of pathogenic strains harboring antibiotic resistance plasmids and in the mechanisms of their elimination. The recent report on gene transfer from plasmid-harboring bacteria to mammalian cells opens the possibility of targeted gene therapy of human diseases (55).

Be that as it may, a wealth of information has been accumulated in recent years on the genetic and functional organization of plasmids, which can now be applied to study the mechanisms involved in the persistence and spread of plasmids in the environment. The incorporation of plasmid-encoded host-killing systems is an interesting feature in the design of microbes to be released into the environment. Knowledge of mechanisms which can specifically hinder plasmid replication, as well as of phenomena underlying plasmid population kinetics, is an important tool for microbial pathologists. In general, we may conclude that although a great deal has been achieved, the new stages in the research of plasmid biology should be based on the confluence of the areas of research outlined here and, ultimately, on the study of plasmids within their natural hosts and in more natural environments.

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The majority of lactococcal plasmids carry a highly related replicon

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DNA sequence analysis and Southern hybridizations, together with complementation experiments, were used to study relationships between lactococcal plasmid replicons. pWVO2, pWVO4 and pWVO5, which co-exist in *Lactococcus lactis* subsp. *cremoris* Wg2, and pIL7 (isolated from another strain) all contained a functional replication region which appeared to be very similar to that of some known lactococcal plasmids. They contain a gene encoding a highly conserved RepB protein (60–80% amino acid identity between pWVO2, pWVO4 and pWVO5), which is essential for replication. When supplied *in trans*, repB of pWVO2 complemented a repB deficiency of pWVO5. Upstream of the repB gene, all these plasmids contain a strongly conserved region including a 22 bp sequence tandemly repeated three-and-a-half times, and an A/T-rich region. The similarity with pWVO2, which is known to replicate via a theta mechanism, suggests that all plasmids of this family are capable of theta replication. Southern hybridizations revealed that many lactococcal strains contain plasmids of this family.

Keywords: lactococcal plasmids, plasmid replication, plasmid incompatibility

INTRODUCTION

Lactococcal strains generally carry a number of different plasmids, varying in size from approximately 2 kb to over 100 kb, some of which specify traits of major interest for the dairy industry. A consequence of the plasmid location is that the desired trait is lost when the plasmid is lost from the population. This is a problem of both fundamental and applied interest. Plasmid loss is a frequently observed problem in Gram-positive bacteria, such as *Bacillus subtilis* (Bron *et al.*, 1991; Gruss & Ehrlich, 1989) and *Staphylococcus aureus* (Novick, 1989), and has also been observed in lactococci (Otto *et al.*, 1983). Recently, we showed that the cloning of large DNA fragments in rolling-circle (RC) plasmids results in poor maintenance in *Lactococcus lactis* (Kiewiet *et al.*, 1993). When the streptococcal theta plasmid pAMβ1 was used for similar experiments, the resulting recombinant plasmids were, however, stably maintained. Based on this observation, we anticipated that endogenous theta plasmids from lactococci would be valuable for the development of stable (food-grade) cloning vectors in lactic acid bacteria.

One of the possible causes of plasmid loss may be the complex mixture of plasmids in most lactococcal strains. It is conceivable that in strains harbouring related plasmids, interaction at the level of replication or partitioning would result in incompatibility. For future development of stable food-grade plasmid vectors it is, therefore, important to analyse the relationships and interactions between plasmids within one strain. In the present work we analysed the replication regions of a number of lactococcal plasmids. Specifically, we addressed the question whether the various plasmids which co-exist in *L. lactis* subsp. *cremoris* Wg2 are related.

A number of lactococcal plasmids, other than those of the RC-type, have recently been analysed. These plasmids, which show a high degree of similarity, comprise the 8.7 kb cryptic plasmid pCI305 (Hayes *et al.*, 1991), the lactose plasmid pSK11L (Jahns *et al.*, 1991), the citrate plasmid pSL2 (Hornig *et al.*, 1991), the 3.8 kb cryptic plasmid pWVO2 (Kiewiet *et al.*, 1993), a 28 kb cryptic plasmid that has been used to construct the nisin-resistant plasmid pVS40 (Von Wright *et al.*, 1990; GenBank accession number L02920), and the bacteriophage-resistance plasmid pCI528 (GenBank accession number L06274). One of these, the cryptic plasmid pWVO2, was derived from *L. lactis* subsp. *cremoris* strain Wg2, which carries a total of five different plasmids (Otto *et al.*, 1983).

Abbreviation: RC, rolling circle.

The EMBL accession numbers for the nucleotide sequences reported in this paper are Z25475 (pIL7), Z25476 (pWVO4) and Z25477 (pWVO5).

The smallest of these, the RC-type plasmid pWVO1 (2·17 kb), has been studied extensively, and a large set of multipurpose cloning vectors is based on it (Leenhouts *et al.*, 1991; Kok, 1991). Of the other plasmids from this strain, denoted pWVO2 (3·8 kb), pWVO3 (7 kb), pWVO4 (19 kb) and pWVO5 (27 kb), only the first has been studied in some detail. Recently, we showed that pWVO2 replicates via a theta mechanism (Kiewiet *et al.*, 1993). Hitherto, no sequence data on the replication regions of pWVO3, pWVO4 and pWVO5 have been available. pWVO5 is of particular interest, since it carries the gene specifying the proteinase required for casein breakdown in cheese production (Otto *et al.*, 1983; Kok *et al.*, 1988). Loss of proteinase activity is frequently observed during cheese production and appears to result from the loss of a plasmid specifying the proteinase gene (Otto *et al.*, 1983).

In this paper we describe the isolation and characterization of functional replication regions of pWVO4, pWVO5 and pIL7. The latter plasmid, extracted from *L. lactis* IL594, specifies a restriction/modification system that renders carrier strains resistant to a number of bacteriophages (Chopin *et al.*, 1984). We were unable to clone the replication functions of pWVO3, and this plasmid was therefore not included in these studies. Since this type of plasmid is closely related to the theta-replicating plasmid pWVO2 (Kiewiet *et al.*, 1993), this family of plasmids is likely to be valuable for the development of stable, food-grade cloning vectors, and for studies on the mechanisms underlying plasmid instability and (in)compatibility in lactococci.

METHODS

Bacterial strains, growth conditions and plasmids. All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were transformed as described by Mandel & Higa (1970) and plated on LB agar or grown on LB medium (Sambrook *et al.*, 1989), supplemented with 100 µg erythromycin ml⁻¹ or 75 µg ampicillin ml⁻¹ when required. Tests for replication proficiency of plasmid derivatives were carried out in *L. lactis* subsp. *lactis* MG1363 (Gasson, 1983). Cells, grown on M17 medium (Terzaghi & Sandine, 1975), were transformed by electroporation (Holo & Nes, 1989) and plated on M17 agar, containing 0·5 M sucrose and 5 µg erythromycin ml⁻¹.

Isolation of plasmid DNA; DNA manipulations. Isolation of plasmid DNA by the alkaline lysis method was essentially as described by Sambrook *et al.* (1989) with some minor modifications. Complete lysis of cells was achieved by resuspending cell pellets in solution I supplemented with lysozyme in a minimal volume of 7·5% of the original volume. This step was followed by an incubation at 55 °C for 10–15 min. For large-scale plasmid isolations, DNA was precipitated with 2-propanol prior to phenol/chloroform extraction in order to reduce the volume. Plasmids were isolated from 1% (w/v) agarose gels using the Prep-a-gene kit as recommended by the manufacturer (BioRad). DNA sequencing was performed by the dideoxynucleotide chain-termination method of Sanger *et al.* (1977). All sequences were determined on both strands. PCR reactions were carried out as described by Sambrook *et al.* (1989). Deletions for determining the region required for replication of

pJRW7 were made with Exo III and mung bean nuclease as described by the supplier (Stratagene).

Oligonucleotides. To sequence the replication regions of pWVO4, pWVO5 and pIL7 a set of ten oligonucleotides was designed. The sequence of these was based on homologous regions between the nucleotide sequences of the *rep* genes of pWVO2, pCI305, pSL2 and pSK11L. Oligonucleotides were constructed in such a way that at locations where different nucleotides were present between the different sequences, these different nucleotides could be incorporated (indicated in parentheses in Table 2), resulting in a mixture of different oligonucleotides in equal amounts. Oligonucleotides P1 through P5 were designed to prime DNA synthesis in one direction; the partly complementary oligonucleotides P6 through P10 were designed to prime DNA synthesis in the other direction. These sets of oligonucleotides enabled us to obtain the sequences for both strands. The position of these primers, relative to the amino acid sequences encoded by the *rep* genes, is indicated in Fig. 1.

Regions outside the *rep* gene were sequenced with oligonucleotides based on the sequences obtained from oligonucleotides P1 through P10 and completed for both strands.

Southern hybridizations. Southern transfers were carried out as described by Sambrook *et al.* (1989) using Gene Screen Plus membranes as carrier (NEN-Research Laboratories). DNA fragments used as probes were prepared by PCR of complementary DNA using primers 1 and 10 (Table 2) and pWVO2 as a template. Probe labelling, hybridization and the detection of complementary DNA were conducted using the ECL gene detection system (Amersham) as recommended by the manufacturer. Hybridization conditions were as described for homologous probes (42 °C, 0·5 M NaCl).

RESULTS

Cloning of replication regions of plasmids pWVO4, pWVO5 and pIL7

Plasmids pWVO4 and pWVO5 were isolated from agarose gels on which the complex plasmid mixture of *L. lactis* subsp. *cremoris* Wg2 was separated. pIL7, originally present in *L. lactis* IL594, was isolated from a lactococcal strain carrying only this plasmid (Chopin *et al.*, 1984). Different approaches were used for the cloning of the replication regions of the various plasmids that were compared in this study.

pWVO4/pJRW4. Plasmid pWVO4 was digested with *Bgl*II. This resulted in three large fragments which were cloned into the *Bgl*II site of pMTL23E (Table 1). Since pMTL23E cannot replicate in *L. lactis*, this vector was used to screen for DNA fragments that enabled replication in this bacterium. Em^R transformants were initially selected in *E. coli* JM101. Only recombinant plasmids which carried the 4·5 kb *Bgl*II fragment from pWVO4, in either orientation, were able to support replication after subsequent transfer to *L. lactis*. One of these plasmids, pJRW4 (Table 1), was used for further analysis.

pWVO5/pJRW5. Plasmid pWVO5 was digested with *Eco*RI. The resulting seven fragments were inserted into the *Eco*RI site of pMTL23E. The ligation mixture was used to transform *L. lactis* directly. Transformants

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Properties	Source or reference
<i>E. coli</i>		
JM101	<i>supE thiΔ(lac-proAB) [F' traD36 proAB lacI^q lacZΔM15</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>L. lactis</i>		
MG1363 subsp. <i>lactis</i>	Plasmid-free, Lac ⁻ Prt ⁻	Lab. collection
subsp. <i>cremoris</i> Wg2	Industrial strain, containing pWVO1-pWVO5	Lab. collection
subsp. <i>cremoris</i> E8L	Natural strain, cryptic plasmid complement	Visser <i>et al.</i> (1986)
subsp. <i>cremoris</i> H61	Natural strain, cryptic plasmid complement	Okamoto <i>et al.</i> (1985)
subsp. <i>cremoris</i> F16	Natural strain, cryptic plasmid complement	Okamoto <i>et al.</i> (1983)
subsp. <i>cremoris</i> IIpl	Natural strain, cryptic plasmid complement	Exterkate (1975)
subsp. <i>cremoris</i> 916	Natural strain, cryptic plasmid complement	Lab. collection
subsp. <i>cremoris</i> Cla2	Natural strain, cryptic plasmid complement	Lab. collection
subsp. <i>cremoris</i> 109	Natural strain, cryptic plasmid complement	Lab. collection
1.10	Natural strain, cryptic plasmid complement	Lab. collection
P8-2-47	Natural strain, cryptic plasmid complement	Lab. collection
9B4	Natural strain, cryptic plasmid complement	Lab. collection
Plasmids		
pWVO2	3.8 kb, cryptic, from <i>L. lactis</i> Wg2	Otto <i>et al.</i> (1983)
pWVO4	6.5 kb, cryptic, from <i>L. lactis</i> Wg2	Otto <i>et al.</i> (1983)
pWVO5	27 kb, specifies proteinase, from <i>L. lactis</i> Wg2	Otto <i>et al.</i> (1983)
pIL7	31 kb, specifies restriction/modification system, from <i>L. lactis</i> II.594	Chopin <i>et al.</i> (1984)
pMTL23E	Ap ^R Em ^R , 4.0 kb, pMTL23 derivative containing the Em ^R gene in the <i>Cla</i> I site (pE194cop6 coordinates 3140-1939) and the T1T2 transcriptional terminator (550 bp) in the <i>Eco</i> RI site; not able to replicate in <i>L. lactis</i>	Kiewiet <i>et al.</i> (1993)
pJRO4	pMTL23E, carrying a 4.5 kb <i>Bgl</i> II fragment of pWVO4, capable of autonomous replication in <i>L. lactis</i>	This work
pJRO5	pBSKII ⁻ , carrying a 7 kb <i>Eco</i> RI fragment of pWVO5, capable of autonomous replication in <i>L. lactis</i>	This work
pJRO55	pMTL23E, carrying a 3.9 kb <i>Hind</i> III fragment of pWVO5, not capable of autonomous replication in <i>L. lactis</i>	This work
pJR7	pMTL23E, carrying a 3.2 kb <i>Sau</i> 3A fragment of pIL7, capable of autonomous replication in <i>L. lactis</i>	This work

selected on erythromycin-containing plates were analysed for their plasmid content. All appeared to contain the same 7 kb *Eco*RI insert, which was found in both orientations. Plasmids containing this insert in one orientation, pJRO5 (Table 1) were used for further analysis.

pIL7/pJR7. In an experiment aimed at the cloning of fragments from large (theta-replicating) plasmids that would increase segregational plasmid stability, a 3.2 kb *Sau*3A fragment of pIL7 was isolated from a partial *Sau*3A digest. This fragment was subsequently cloned into the *Bam*HI site of pMTL23E. Plasmid DNA containing this insert was isolated from *E. coli* and used to transform *L. lactis*. Plasmid-containing transformants were obtained,

indicating that the 3.2 kb insert carried a region of pIL7 that was capable of sustaining autonomous replication in *L. lactis*. This plasmid, pJR7 (Table 1), was used for further analysis.

DNA sequences of the replication regions of pJRO4, pJRO5 and pJR7

Based on the similarities between pWVO2 and several other previously described lactococcal plasmids (Hayes *et al.*, 1991; Jahns *et al.*, 1991; Horng *et al.*, 1991; Kiewiet *et al.*, 1993) we speculated that most lactococcal plasmids, other than those of the RC-type, might contain highly related, functional theta-type replication regions (Kiewiet *et al.*, 1993). Support for this idea was obtained from

Table 2. Oligonucleotides used for sequencing of replication genes from pWVO4, pWVO5 and pIL7

Nucleotides in parentheses indicate different possibilities at that location.

Oligo	Sequence	Readable sequence obtained in the dideoxy chain-termination reaction		
		pWVO4	pWVO5	pIL7
P1	AA(TA) CAA AAG CAG GTG C	—	—	+
P2	ATG CAA (GA)A(GA) CAA GC(CT) TTT T	—	+	+
P3	GAA (TC)TA (TC)AA CCA ATA CG	—	—	+
P4	(CT)AT TGT CTT TCA TAT	+	+	+
P5	GG(GT) G'TC AAA GAC CAC TTG TC	+	+	+
P6	GAC AAG TGG TCT TTG AC(AC) CC	+	+	+
P7	(GA)AT ATG AAA GAC AAT (GA)	+	+	+
P8	CGT ATT GGT T(AG)T A(AG)T TC	—	+	+
P9	AAA A(GA)G CTT G(CT)T (CT)TT GCA T	+	+	+
P10	CAA GGT (TCA) (TA) (GA) CAC CTG CTT TT	+	+	+

Southern hybridizations in which the *repB* gene of pWVO2 was used as a probe against the entire plasmid content of *L. lactis* subsp. *cremoris* Wg2. This resulted in cross-hybridizations with pWVO2 as well as pWVO3, pWVO4 and pWVO5 (results not shown). To study whether pJRO4 and pJRO5, which were obtained from pWVO4 and pWVO5, as well as pJR7, contained replication regions similar to that of pWVO2, a set of ten oligonucleotide primers (Table 2) was designed on the basis of conserved regions in the nucleotide sequences of the *rep* genes of pCI305, pSK11L, pSL2 and pWVO2. With most of the oligonucleotides, clear sequence ladders were obtained (Table 2). To complete the sequence for both strands, additional oligonucleotides, based on the sequences obtained, were used. Analysis of the resulting sequences in each case revealed an ORF, similar to that of the *rep* genes of the plasmids from which the oligonucleotides were designed. Insertion of random λ DNA fragments into the *Sna*B I site in this ORF of pJRO4, or the *Hind*III site in the corresponding ORF of pJRO5, abolished replication in *L. lactis*. The minimal region required for replication of pJR7 was determined by creating deletions from either side of the cloned fragment with Exo III and mung bean nuclease and subsequent transformation to *L. lactis*. Deletions in one direction were created using the *Sac*I site as the 3' protected end and the *Eco*47-3 site as the 5' starting point for Exo III. Deletions in the other direction were created with the *Dra*III site as the 3' protected end and the *Mlu*I site as the 5' starting point. The deletion end-points and the entire upstream region of the pIL7-derived ORF of pJR7 were sequenced using the M13 universal and reverse primers (Short *et al.*, 1988). Replication of pJR7 in *L. lactis* was abolished when deletions removed the last 153 nucleotides from this ORF. These results show that the respective ORF in each of the analysed replicons is required for replication.

By analogy with similar regions of pCI305, pSK11L, pSL2 and pWVO2, the described ORFs of pJRO4, pJRO5 and pJR7 were denoted *repB*. In Fig. 1 the deduced amino acid sequences of the RepB proteins of pWVO2, pWVO4, pWVO5 (from strain Wg2) and pIL7 (from strain IL594) are compared with those of the equivalent proteins of other replicons. The sequences show an overall identity of approximately 30%. Conservation was extremely high in the N-terminal and central regions. The levels of identity are even higher for plasmids of certain subgroups. For instance, the levels of identity amount to 60–80% for the RepB proteins of pWVO2, pWVO4 and pWVO5, which are derived from the same strain.

Additional sequences of the region upstream of *repB* of pWVO4 and pWVO5 were determined by primer walking using oligonucleotides based on the sequences obtained. The sequence of this region of pJR7 could be determined with universal and reverse primer by using deletion derivatives, obtained from the Exo III and mung bean nuclease reactions. The corresponding regions of pWVO2 (Kiewiet *et al.*, 1993) and pCI305 (Hayes *et al.*, 1991) have previously been shown to be required for replication. Sequences similar to those determined for the previously mentioned lactococcal plasmids were found (Fig. 2). Both contain a 22 bp sequence tandemly repeated three-and-a-half times. Comparison of all repeats revealed a consensus sequence, reading 5' ANN(G/C)(T/C)N(T/A)NAAAA-AAC(T/G)(G/A)TGTAT-3'. In addition, two regions of dyad symmetry, overlapping the putative promoter region, were identified. The inverted repeat downstream of the -10 region is highly conserved in all three pWVO2-related plasmids of *L. lactis* subsp. *cremoris* Wg2, as well as in most other lactococcal plasmids described. The region upstream of the 22 bp repeats carries a number of AT-rich stretches separated by GC-clusters. The

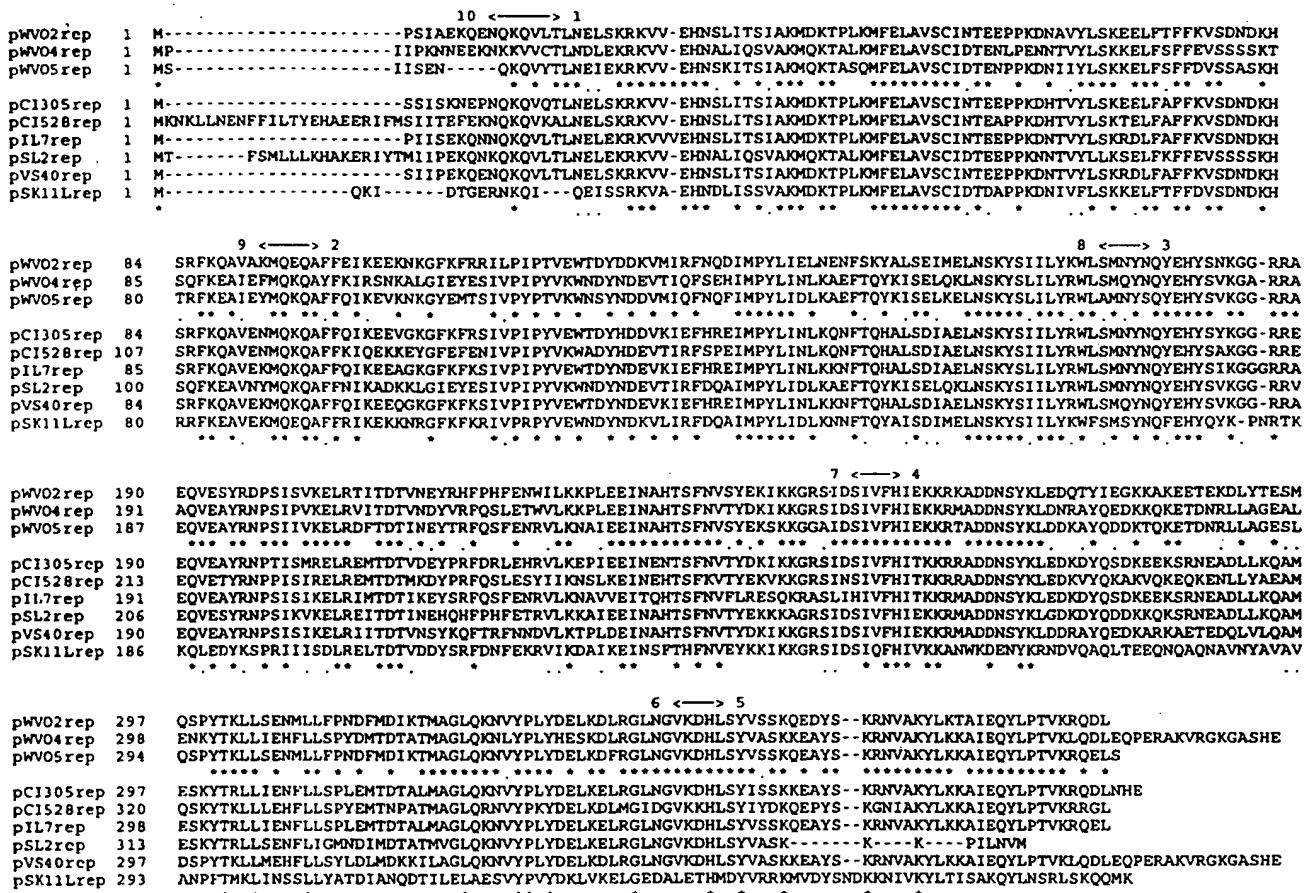


Fig. 1. Alignment of deduced amino acid sequences of RepB proteins specified by lactococcal plasmids pWVO2 (Kiewiet *et al.*, 1993), pWVO4, pWVO5, pCI305 (Hayes *et al.*, 1991), pCI528 (GenBank accession number L06274), pIL7, pSL2 (Hornig *et al.*, 1991), pVS40 (GenBank accession number L02920) and pSK11L (Jahns *et al.*, 1991). The one-letter code is used. Numbers to the left of the sequences indicate the position of the first (N-terminal) aa residue of each protein. The first three plasmids (pWVO2, pWVO4 and pWVO5) were derived from the same strain (*L. lactis* subsp. *cremoris* Wg2). Identical amino acids in all sequences are indicated with an asterisk, similar amino acids with a dot. The relative positions of the primers listed in Table 2 are indicated above the sequence. Directions for primer extension are indicated by arrowheads.

nucleotide sequences of these stretches show a very high level of conservation (Fig. 2). As for pWVO2 and pCI305, this region was determined to be part of the minimal region required for replication of pJR7. Replication of derivatives obtained from the Exo III and mung bean nuclease reaction was abolished when deletions extended within this region. The smallest derivative that still supported replication extended 134 bp upstream of the region as represented in Fig. 2.

Presence of an additional ORF downstream of repB

Immediately downstream of the *repB* gene, the start of another ORF could be detected in the sequences of pJRO4, pJRO5 and pJR7. The entire sequence of this ORF was determined for pJR7 and found to encode a putative protein of 208 amino acids (Fig. 3a). The putative start codon (ATG) of this ORF, denoted ORF X, overlaps with the stop codon (TGA) of the upstream *repB* gene, which is indicative of translational coupling between these genes. A similar start can be identified in the related

ORF X sequences of most other lactococcal plasmids (Fig. 3b). In the sequence of pWVO2 only the first 14 codons of ORF X are present. The start of ORF X in all the plasmids analysed is preceded by an appropriate Shine-Dalgarno sequence (Van de Guchte *et al.*, 1992). All deduced amino acid sequences were highly similar and showed an α -helix-turn- α -helix motif between amino acid residues 6 and 28, indicating a DNA-binding potential for this region. Deletion of this ORF from pJR7 by the Exo III and mung bean nuclease method did not affect replication. No putative transcriptional terminator could be identified downstream of either *repB* or ORF X. The observation that ORF X can be deleted from pJR7 without affecting replication, and the fact that this ORF is almost completely absent from pWVO2, suggest that it is not essential for replication.

repB of pWVO2 can complement repB of pWVO5

A 3.9 kb *Hind*III fragment of pJRO5, contained entirely within the 7 kb *Eco*RI fragment of pWVO5, which carried

		
pWVO2	ACAAGTTTAAAGCCTAT-ATCT	ATTTATCAT-ATATATTTAATCTTT	A	T-CTTTTG	-CGTAAAAAAA-GTCAGTGGTAGCAAGGGTATGCAGAATTAAAC		=
pWVO4	AAAAAAAGCGAAGCCTAT-ATCT	ATTTATCAT-ATATATTTAATCTTT	G	TTCTTTTG	GGGTGCTAAAAAAATCAGTGTTCAGGTTATAAGATTTTAT		
pWVO5	AAAAAAAGCGAAGCCTATTATAT	ATTTATCAT-ATATATTTAATCTTT	G	TTCTTTTG	GGGTGAAAAAAAGTCAGTGGTAGCAAGGGGAGCAGATTAT		
pIL7	AAAAAGGCCAGC-TATTATAT	ATTTATCAT-ATATATTTAATCTTT	G	TTCTTTTG	-CGTAAAAAAA-GTCAGTGTATAGGTTATCCAATTAT		
pCI305	AAAAATAGCGAAGCCTATTATAT	ATTTATCTT-ATATATTTAATCTTT	A	TTCTTTTG	-CGTCAAAAAAA-TCAATATTTCAAGGTTATAGAATTAT		
pCI528	AAAAAAAGCGAAGCCTAT-ATTA	ATTTATCAT-ATATATTTAATCTTT	C	TTCTTTTG	-CGTCAAAAAAA-GTTAGTATTTAAGGGTTACAGAATAAT		
pVS40	AAAAAAAGCGAAGCCTAT-ATTA	ATTTATCAT-ATATATTTAATCTTT	A	TTCTTTTG	-CGTGGAAAAAA-GTCAGTGTAAAGGTGGATACAGAATTATAG		
PSL2A	AAAAAAAGCGAAGCCTATTATAT	ATTTATCAT-ATATATTTAATCTTT	G	TTCTTTTG	-CGTGAaaaaaa-GGCAGTGTTCGCTAGTTATAGAAATTAAAC		
PSK11L	ACTCCTAGAGTCCTTTAATT	ATTTATATATATATATTTT	G	TTCTTTTG	CGAAAAAAATCTAGTGTGCAAGGGTAACAGGATTATAG		

-35 <-

>>>						<-<-<-	
PWVO2	AGTCAGAAAAAACTGTGTATACAGTCAGAAAAAACTGTGTATACAGTCAGAAAAAACTGTGTATACAGTCAGAAAAA-						-ATTGAAACACT
PWVO4	AGCAAGAAAAATCGATGTATAGCAAGAAAAATCGATGTATAGCAAGAAAAATCGATGTATAGCAAGAAAA-						-TTGCAGAAAA
PWVO5	AGCAACAAAAAACGATGTATAGCAACAAAAACGATGTATAGCAACAAAAACGATGTATAGCAACAAAA-						-ACTTGATTATGT
PIL7A	AGCAACAAAAAACTGTGTATAGCAACAAAAACTGTGTATAGCAACAAAAACTGTGTATAGCAACAAAA-						-AATTGCTGTGT
PCI305	ACCAACAAAAAACTGTGTATATACCAACAAAAACTGTGTATACACCAACAAAAACTGTGTATACACCAAA-						-CTTCTTTGTT
PCI528	AGCATAAAAAAACTGTGTATAGCATAAAAAAACTGTGTATAGCATAAAAAAACTGTGTATAGCATAAAAAA-						-ATCATCATTTA
PVS40	CGTATGAAAAAACTGTGTATAGCGTATGAAAAAACTGTGTATAGCGTATGAAAAAACTGTGTATAGCGTATGAAAAA-						-ACTTGATAGATA
PSL2	AGTCACAAAAATCGATGTATACAGTCACAAAAATCGATTTGTGACTCTATGCATCGATGTATACGAGTCACAAAAATCGATGTACACAGTACGACTTTGTATTTGT						
PSK11L	TCCTACAAAAAACTGTGCATAGTCCTACAAAAAACTGTGTATAGTCCTACAAAAAACTGTGTATAGTCCTACAAGTT-						-ATTTGTTGTTG

-10

RBS

>>>						<-<-<-	
PWVO2	GAATCGCTTTGATATAATAAAAA	-	-	GCATAGAGAAATTGACTCGCTAAAGATTCTCTATGCCTATTTAAAAACACTCAC	-	-AAAGGAGTATTA	-CTATG
PWVO4	CTAATTTCTGATATAATAAAA	-	-	GCATAGAGAAATCACAGCTAAATGAATTCTCTATGCCTAGACTAAA	-CACGC	-AAAGGAGCG	-TATG
PWVO5	TGCTATAAATTGTATAATGA	-	-	AAGCATAGAGAAAATTGACGT	-CTAAATCACTTCCTATGCCTAAATCACAAACACTCACAAAGGAGTATT	-	-CTATG
PIL7	TGCTATAATGTGGTACAATAAAA	-	-	GACATAGAGAACTATCCCGCAAAGAATCT	-TTCTCTATGCCTACTTATAA-CACGC	-AAAGGAGCGTATT	-CATG
PCI305	TGTTTCGTTGGTATATAATGATATAATAAAGCATGAAGAACTCTCTACGAAAAGTGT	-	-	TTCTCTATGCCTATCTAAACTCACTCAC	-AAAGGAGCAGTT	-	-CTATG
PCI528	TGCTATATTATGATATAATAA	-	-	AAGTATGAGAGAACAAACTTTGAAACGAGATTCTCTACTTATGAAACACGC	-AGAGGAGCGTATCTT	-	-TATG
PVS40	TACGGTATTCTGATACAATAAAA	-	-	GCATAGAGAAATCGACAG	-CGAAATCAGTTCTCTATGCCTACTTAAA	-TCACTCACAAAGGAGTAAC	-CTATG
PSL2	GTACTGTATAGTATAATAAA	-	-	AGCATAGAGAAAACACTCACTATGAAATAC	-TTCTCTATGCCTACTTAAACACGC	-AAAGGAGCGTATT	-TATG
PSK11L	AGGTGTTCTGTTATTAT	-	-	TTATTTAA	-TCA-T-A	-AAAGGAGTGGAT	-TATG

Fig. 2. Alignment of the region upstream of *repB* of the plasmids described in Fig. 1. Regions with strong sequence conservation are boxed. GC-regions, flanking AT-rich regions, are indicated with asterisks. The 22 bp sequences repeated three-and-a-half times are indicated by double arrows. Inverted repeat regions are indicated by dashed arrows. Putative transcription and translation signals are indicated in bold and by single lines.

the region upstream of the *repB* gene and the first 85 codons of the *repB* gene, was cloned into the *Hind*III site of pMTL23E and propagated in *E. coli*. This upstream region is essential for replication of pCI305 (Hayes *et al.*, 1991) and pWVO2 (Kiewiet *et al.*, 1993) as well as pJR7. The resulting plasmid, denoted pJRO55, was no longer capable of autonomous replication in *L. lactis*. pJRO55 was then used to transform an *L. lactis* strain carrying only pWVO2. As a control, pJRO5 was used to transform pWVO2-containing *L. lactis* cells. Transformants were selected on M17 agar supplemented with erythromycin. The transformation efficiency with pJRO55 was about 10-fold lower than with pJRO5. Several erythromycin-resistant transformants were analysed for their plasmid content. pJRO5 could clearly be detected on agarose gels in small-scale plasmid isolations (1.5 ml culture) of *L.*

lactis, while pJRO55 could only be detected using large-scale plasmid isolations (100 ml culture). On the basis of ethidium bromide fluorescence intensities on agarose gels we estimated the copy number of pJRO55 in these cells to be less than one per chromosome equivalent. To verify that plasmid pJRO55 was intact and that Em^R transformants were not the result of recombination between pWVO2 and pJRO55, plasmid DNA from several erythromycin-resistant transformants was isolated and used to transform *E. coli*. Ten of the transformants were analysed for their plasmid content. All appeared to contain intact pJRO55. These results indicate that the *repB* gene of pWVO2, when supplied in *trans*, can support the replication of pJRO55, which lacks a functional copy of its own *repB* gene. In addition, this experiment provides further proof that the upstream region of *repB* is required

(a) ORF X pil7

ATGAGTGAAAGTTAAAGACAATGAAAGAGCTGCAGATGAGTTGAGTGTACAAAAGAAAATTCAATATCACTACCAAGGGTACCAA
 M M S E K L K T M K E L A D E L S V T K E N I Q Y H Y Q R L P
 start
 AGAATTACAGATTAAACCGTTCCAATGGGTCTAATCTAATAAATTCTAAAGCAGAAAAATAATTAGTAAAGTAGAAAGTAGCAA
 K E L Q I K T R S N G S N L I N S K A E K I I L G K V E S G S K
 ATCAAATACAAAAGCCAACAAATAAGTAGCAAAGAAAATTGAGATGAAAATTAGATAATCCCTTATTAATAGATTAATCAAAGAGTT
 S N T K D Q Q I S S K E N F E D E K L D N P F I N R L I K E V
 GAGTATTGAAAATGACAAGGATAAAATCATATCTACCAAGACCAACAAATAAGTAGCAAAGACCAACAAATAGAAAACTAACTAATTGTT
 E Y L K I D K D K I I S T K D Q Q I S S K D Q Q I E K L T N L
 TAGACCAGCAACAAACGTTAGCCTTCAGGATAAAAGTTACTGATGAGTATAAGTCAGAAATCAATGACTAAAAGCTCTAAATGCCAAC
 L D Q Q Q R L A L Q D K K L L D E Y K S E I N D L K A L K M P T
 ACAGGAAACAGAATTCAAACACTTAGACAATCAATATAAGATGAAGTGAACGCTCTTAAAGAGAAGTTGAAAATTTCAGGAACAAATCAA
 Q E T E F K H L D N Q Y K D E V N A L K E K L E N F Q E Q I K
 GTTCAAAAAGGATAGAAGAACAAAGAAAACCAAGAAAGTGGTGGGACTATGGCGAAAATAG
 V Q K R I E E Q E K P R K W W G L W R K end

(b)

pWVO5 -----
 CTACCTACGGTTAAAGGCAGGAATTATCATGAGTGAGAATTAAACTATTAAGGAGCTT
 L P T V K R Q E L S ——————
 M S E N L K T I K E L

pWVO4 -----
 TGCAAAGGTTCGAGGTAAGGTCAAGCCATGAGTGAGAATTAAACGATTAAGGAGCTT
 A K V R G K G A S H E ——————
 M S E N L K T I K E L

pWVO2 -----
 TACCTACCAACGGTTAAAGGCAGGACTTATGAGTAGTGAAGAAAGTAAAGACAATCAAAGAG
 Y L P T V K R Q D L ——————
 M I S E K L K T I K E

pIL7 -----
 TATCTACCTACGGTTAAAGGCAGGAATTATGAGTGAGTGAAGAAAGTAAAGACAATGAAAGAG
 Y L P T V K R Q E L ——————
 M M S E K L K T M K E

pCI305 -----
 ACCTACGGTTAAAGGCAGGACTTAAACCATGAGTGAGAACTTAAACGATTAAGGAGTG
 P T V K R Q D L N H E ——————
 M S E N L K T I K E L

pCI528 -----
 CTACCAACGGTTAAAGGCAGGACTTAAACCATGAGTGAGAATTAAACGATTAAGGAGCTA
 Y Q R L K G G D Y N E ——————
 M S D N L K T I K E L

pVS40 -----
 TGCAAAGGTTCGAGGTAAGGTCAAGCCATGAGTGAGAATTAAACGATTAAGGAGCTT
 A K V R G K G A S H E ——————
 M S E N L K T I K E L

pSL2 -----
 GCCAACGGTTAAAGGCAGGACTTAAACCATGAGTGAGAAGACTTAAACGATTAAGGAGTTG
 P T V K R Q D L N H E ——————
 M S E D L K T I K E L

for replication. In the absence of selective pressure pJRO55 was rapidly lost from the culture, whereas pJRO5 was stably maintained. Loss of pWVO2 was never observed.

Southern hybridizations of plasmids from various lactococcal strains

Hayes *et al.* (1991) showed that the type of replicon described here is widespread among lactococci. We tested an additional set of ten *L. lactis* strains for the presence of plasmids with a similar replicon. A 1 kb PCR fragment of the replication gene *repB* of pWVO2, obtained with primers 1 and 6 (Table 2), was used as a probe. At least

one plasmid in each strain tested was found to hybridize to this fragment (Fig. 4).

DISCUSSION

In the present work we have studied relationships between lactococcal plasmids which are not of the RC type. The most significant conclusions are: (1) that many of these plasmids contain a functional replication region that is part of a family of highly related replicons; and (2) that different plasmids containing these replication regions co-exist in one and the same *Lactococcus* strain. We showed that at least three out of five different plasmids present in *L. lactis* subsp. *cremoris* Wg2 contain a functional replication region of this family. One of these plasmids

Fig. 3. (a) Complete nucleotide sequence of ORF X of pil7. The deduced amino acid sequence is presented below the sequence in the single-letter code. (b) Probable translational coupling between the *repB* genes of eight plasmids and ORF X. The putative Shine-Dalgarno sequence is indicated by a dashed line above the sequence. The stop codon of the *rep* gene is indicated with a solid line below the sequence. ORF X of pWVO2 ends after the 14th codon. From the nucleotide sequence of pSL2, as obtained from GenBank (accession number X56550), the ORF encoding *repB* ends at the position indicated in Fig. 1. Deletion of a single base in the 3' region of the nucleotide sequence of this gene, however, would extend the ORF to the location indicated here. In that case, ORF X would also be translationally coupled to the *repB* gene.

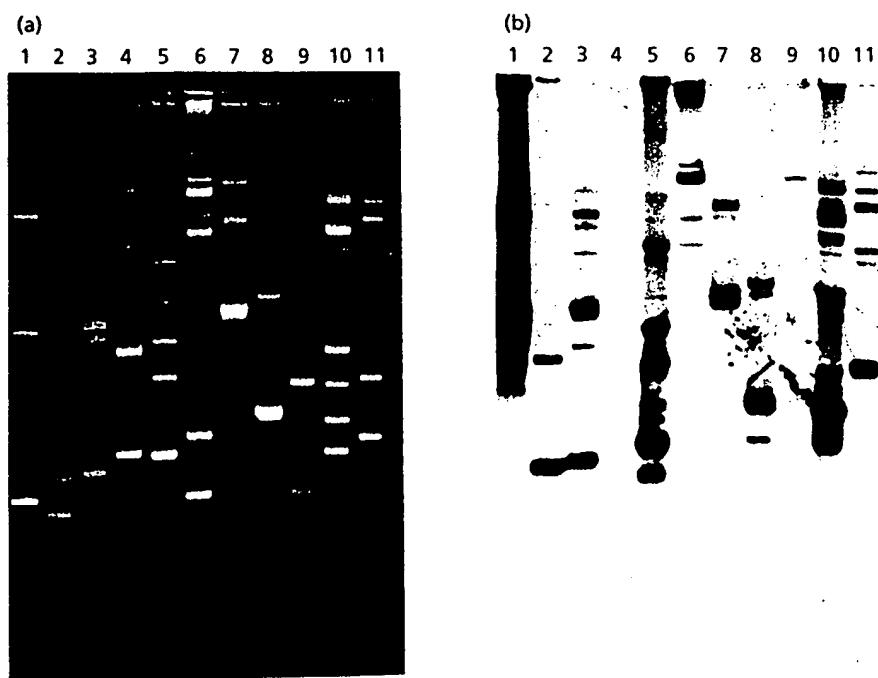


Fig. 4. Distribution of the *repB*-like replication gene in a number of lactococcal strains. (a) Plasmid profiles of a number of natural *Lactococcus* strains: 1, *L. lactis* subsp. *cremoris* Wg2; 2, *L. lactis* subsp. *cremoris* H61; 3, *L. lactis* subsp. *cremoris* F16; 4, *L. lactis* subsp. *cremoris* Hpl; 5, *L. lactis* subsp. *cremoris* E8L; 6, *L. lactis* subsp. *cremoris* Cla2; 7, *L. lactis* subsp. *cremoris* 109; 8, *L. lactis* subsp. *lactis* L10; 9, *L. lactis* subsp. *cremoris* 916; 10, *L. lactis* subsp. *lactis* P8-2-47; 11, *L. lactis* subsp. *lactis* 9B4. (b) Fluorograph of Southern hybridization of the DNA in (a) with the 1 kb PCR fragment obtained with primers 1 and 6 of the *repB* gene of pWVO2.

(pWVO2; Kiewiet *et al.*, 1993) has been shown to replicate via a theta mechanism. This suggests that the other members of this plasmid family can replicate via a similar mechanism. The results obtained do not rule out the possibility that additional functional replication regions might exist on pWVO4, pWVO5 or pIL7. We consider this possibility unlikely, however, since with none of the restriction digests used here (including a partial *Sau*3A digest of pIL7) were alternative replicons observed.

The replicons studied in this work, and other plasmids of this family, can be divided in two segments. One segment contains the *repB* gene, encoding the RepB protein, which is essential for replication of pC1305 (Hayes *et al.*, 1991) and pWVO2 (Kiewiet *et al.*, 1993). The three plasmids analysed in the present studies also require an intact *repB* gene for replication. As shown for pC1305 and pWVO2, the other essential segment is the non-coding region (approximately 300 bp) upstream of *repB*. By analogy with Gram-negative theta-replicating plasmids, like RSF1010 (Frey *et al.*, 1992) and RK2 (Perri *et al.*, 1991), for which it has been established that a number of direct repeats are required for binding of the Rep protein, it is conceivable that the 22 bp sequence repeated three-and-a-half times also constitutes the binding region for the RepB protein in theta-replicating lactococcal plasmids and that this binding is required for initiation of replication. In this context, the observation that pWVO2 can complement a RepB deficiency of pJRO5 is of interest. This can be explained by assuming that RepB of pWVO2 can recog-

nize the binding region of pJRO55 and initiate replication. Since RepB is also required for replication of pWVO2 and probably has a higher affinity for its cognate binding sequence, pJRO55 will be maintained at a much lower level and rapidly be lost in the absence of selective pressure. Differences in the sequence of the 22 bp repeats of plasmids of this type are likely to determine the difference in affinity of RepB. This may explain why pWVO2 and pJRO5, which carry related *repB* genes and related 22 bp repeats, can co-exist within one host strain. Affinity studies, currently in progress, between the replication proteins from this family of replicons with the different repeated sequences should give more insight into the phenomenon of plasmid (in)compatibility within one strain.

The region upstream of the 22 bp repeats is highly conserved. Deletion analysis of pWVO2 has shown that this region is required for replication (Kiewiet *et al.*, 1993). On the basis of its high AT content, this region could be the site of strand separation from which replication starts. This does not, however, require strong conservation of the nucleotide sequence. Possibly, this region is the recognition site for host-encoded functions involved in replication, such as DnaB- and DnaC-like proteins, which are known to be involved in the replication initiation of a number of theta-replicating *E. coli* plasmids (Bramhill & Kornberg, 1988).

The function of ORF X, the expression of which is probably translationally coupled to that of *repB*, is as yet

unclear. In pWVO2, the putative ORF X product will be truncated since a stop codon is present after codon 14, which is followed by a transcriptional terminator within 100 bp (Kiewiet *et al.*, 1993). No sequence errors are likely to be present in this region. The likely translational coupling of ORF X to *repB* and the deduced DNA-binding properties of the ORF X product suggest an involvement in replication. Since, however, ORF X is not present intact in pWVO2, and can be deleted from pJR7 without preventing replication, its presence is not essential.

Only a limited number of lactococcal plasmids did not hybridize to the pWVO2 *repB* probe. A number of smaller plasmids hybridized with a probe derived from pWVO1 (results not shown). The latter RC replicon is also widespread among lactococci, but the strains tested so far by us and others (Dr A. Geis, personal communication) never contained more than one member of this family of plasmids. Although we have observed that a few plasmids do not hybridize to either probe (results not shown), and these might constitute yet another family of plasmids, the present results indicate that only two families of plasmid replicons are common in lactococci: the first family contains members of the pWVO2-type theta replicons, and the second, members of the pWVO1-type RC replicons.

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